WOUNDING AND ULTRAVIOLET RADIATION STRESSES AFFECT THE PHENOLIC PROFILE AND ANTIOXIDANT CAPACITY OF CARROT TISSUE

A Dissertation

by

BERNADETH BIDARIMURTI SURJADINATA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Food Science and Technology

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ABSTRACT

Wounding and Ultraviolet Radiation Stresses Affect the Phenolic Profile and Antioxidant Capacity of Carrot Tissue. (May 2006)

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Abiotic stresses have been known to induce the production of secondary metabolites in plants as part of the defense mechanism systems. Many of these compounds were reported to have bioactive properties, such as antioxidant, antimutagenic, antimicrobial, or anticarcinogenic. By using these stresses as tools, it is possible to develop strategies to produce fruits and vegetables with higher health promoting compounds.

We determined that wounding and UV radiation caused total phenolics, antioxidant capacity (AOX), and PAL enzyme activity to increase significantly. This increase is affected by wounding intensity, time period of UV exposure, and duration of storage. There was no significant difference between the cultivars tested (Navajo, Legend, and Choctaw).

The main phenolic compounds identified for all cultivars using HPLC-DAD were chlorogenic acid and its isomers (dicaffeoylquinic acids), ferulic acid, *p*-hydroxybenzoic acid and its derivative, *p*-coumaric acid derivative, and isocoumarin. Combining wounding with different UV lights (A, B, and C) induced the production of different phenolic compounds. The type of phenolics induced depended on the type of UV radiation used. The synthesized phenolic compounds showed a high linear relationship with AOX capacity. The specific antioxidant activity induced by each stress treatment depended on the induced phenolic profile.

Reactive oxygen species (ROS), jasmonic acid (JA), and ethylene are all involved as secondary signaling molecules in the wounding and UV stress-induced phenolic metabolism. This is shown by the decrease in the synthesis of total phenolic compounds in stressed carrot tissue when using diphenyleneiodonium chloride (DPI), phenidone, or 1-methylcyclopropene (1-MCP), which inhibit NADPH oxidase (ROS production), lipoxygenase (key enzyme in JA metabolic pathway), and block ethylene receptor, respectively. These inhibitors showed a dose response effect by reducing the synthesized phenolic compounds. This information, plus the combined effects of the inhibitors enabled us to propose a possible signaling mechanism of stress-induced phenolic synthesis caused by wounding and different UV radiations.

Abiotic stresses, such as wounding and UV radiation, elicit the synthesis of phenolic compounds, increasing the antioxidant capacity of the tissue. This approach shows the potential of using plant stress-induced signaling mechanisms as tools to manipulate the synthesis of desirable phenolic compounds or other secondary metabolites.

DEDICATION

To my beloved father, Djohan Surjadinata, a great man who challenged and convinced me to pursue this degree.

Without his words of wisdom, I would not be the person that I am today.

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Last, but not least, I would like to thank God for giving me many blessings and strengths. Thank you for allowing me to achieve this goal and for giving me a wonderful life.

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CHAPTER I

INTRODUCTION

In recent years, consumers prefer food products that are not only healthy but also ones that can provide convenience. Fresh-cut fruits and vegetables are excellent examples of products that can offer both. Fresh-cut produce is also known as ready-to-eat or minimally processed. They are usually trimmed, peeled, and/or cut, if necessary washed, and sometimes disinfected.

Since they are not blanched or subjected to heat treatment, these fresh-cut fruits and vegetables are still living tissues (Shewfelt 1986, Rolle and Chism 1987). This means that they are still going through continuous physiological activity, including the synthesis of health promoting compounds. Many of these nutraceutical compounds, such as phenolics, vitamins C and E, and carotenoids are antioxidants that have been associated with prevention of cancer, cardiovascular diseases, diabetes, cataract, arthritis, aging, and neurological diseases (Deshpande and others 1996).

If the nutraceutical content of fresh-cut fruits and vegetables can be enhanced, then these products will have potentially greater health benefits and higher market value. There are other several approaches that can be used to enhance the accumulations of desirable bioactive compounds, such as plant breeding, optimizing the preharvest practices, or genetic engineering (Cisneros-Zevallos 2003). One alternative strategy is to increase the nutraceutical content by applying postharvest abiotic stresses such as wounding and UV radiation, either alone or using a combination of both. These stresses can be used as tools to increase the amount of phenolics that posses bioactive properties.

When stress occurs, plant cell can activate specific transcriptional genes (Saltveit 2000, Leon and others 2001).

This dissertation follows the style and format of Journal of Food Science.

The roles of these genes are to adjust the plant metabolism to repair and heal the damage, and also to produce substances to prevent invasion by predators. These processes could occur between a few minutes to several hours after wounding (Leon and others 2001).

Most plants, if not all, contain phenolic compounds. These compounds form one of the main classes of secondary metabolites. Phenolics are formed by two metabolic pathways: shikimic acid and malonic acid pathways. The most abundant classes are derived from phenylalanine and catalyzed by PAL enzyme. Their chemical structure has an aromatic ring bearing one or more hydroxyl group, including their functional derivatives (Shahidi and Naczk 1995).

Phenolics found in foods generally belong to phenolic acids, flavonoids, lignin, stillbenes, coumarins, and tannins (Harborne 1993). Many of these are soluble in water or organic solvents. Phenolics affect food systems in two different ways. First, they are greatly involved in the sensory quality, such as color, taste and aroma. At high concentrations, phenolics may participate in discoloration of foods, interactions with proteins, carbohydrates, minerals, and also may contribute to astringency and bitterness flavor. On the other hand, some phenolics possess bioactive properties that are very beneficial for human health, such as acting as antioxidants (Alasalvar and others 2001).

Phenolic antioxidants have a primary function as free radical scavengers and terminators by donating hydrogen atoms or electrons (Robbins 2003). The result of this reaction is a stable phenoxy radical (Nawar 1985). This is due to resonance delocalization of unpaired electrons around the aromatic ring and lack of suitable sites for attack by oxygen molecule (Nawar 1985, Shahidi and Wanasundara 1992). In general, the more available hydroxy groups the phenolic has, the more potent it is as antioxidant and to be a successful antioxidant, the phenoxy radical formed after donating the hydrogen atom, must not produce further radicals but must rather be a relatively stable species (Robards and others 1999).

To be able to survive and reproduce, plants have to be capable to handle different potentially harmful stress conditions that occur almost constantly in their environment (Stratmann 2003). When plants are subjected to stress, they will activate their stress-related defense genes. The functions of these genes are to neutralize the stress, repair damage, produce compounds to inhibit growth of predator insects, participate in the activation of the defense signaling pathways and adjust the plant metabolism to the nutritional demands (Leon and others 2001, Stratman 2003).

Wounding will cause some physiological effects on the plant tissue and it is understood that the greater the degree of cutting, the greater the wounding response will be (Surjadinata and Cisneros-Zevallos 2003). Most of these effects are known to decrease the quality of the produce. By cutting, the tissue will produce more ethylene (C_2H_4), have higher respiration (Surjadinata and Cisneros-Zevallos 2003), lose more moisture, and cause membrane deterioration (Toivonen and DeEll 2002). All of these responses will eventually cause the produce to have a shorter shelf life. Controlling these responses is a major obstacle that must be overcome since it is the key to providing a minimally processed product of good quality (Cantwell 1992).

Change in phenolic contents is one of the most studied phenomena in response to wounding (Toivonen and DeEll 2002). Recently, some studies have shown that wounded tissues have higher phenolic content and higher antioxidant capacity. Heredia and Cisneros-Zevallos (2002) found that wounding stress increased the phenolic content and therefore increased the antioxidant capacity of carrots. These findings were also shown in other tissues, such as lettuce leaf (Kang and Saltveit 2002) and purple-flesh potato (Reyes and Cisneros-Zevallos 2003). However, all of these studies were only done with one type of cut, such as slices. There is a need to quantify the change in phenolic content and antioxidant capacity under different wounding intensities.

UV radiation can be divided into three parts: UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (200-280 nm). UV-A is the least harmful part of UV radiation. UV-B can cause some damaging effects in plants and UV-C is the most dangerous to any

organisms; however the stratospheric ozone layer filters out most of the UV-C radiation (Hollosy 2002).

Recently, it was found that UV radiation induces the expression of several plant defensive genes that are normally activated after wounding (Conconi and others 1996, Stratmann 2003). These two stresses, wounding and UV radiation, trigger the production of jasmonic acid (JA), ethylene (C_2H_4), and reactive oxygen species (ROS) (Conconi and others 1996, Hollosy 2002, Rakwal and Agrawal 2003, Stratmann 2003). It is still unknown if they are dependent or independent of each other. By using elicitors that can inhibit a specific pathway, it would be possible to obtain a better understanding about the mechanisms of these signaling molecules related to the synthesis of phenolic compounds.

The overall goal of this study was to determine the effect of wounding and UV radiation stresses on the synthesis of phenolic compounds, antioxidant capacity and PAL activity. This dissertation has been divided into three main chapters. In chapter II, we investigated the effect of wounding stress on carrot tissue on total and individual phenolics, antioxidant capacity, and PAL activity. Different wounding intensities, storage times and carrot cultivars were analyzed. The result from this experiment was used to determine the amount of storage time needed for the carrots to accumulate the maximum phenolic content and antioxidant activity. Total phenolics, AOX capacity and PAL activity were measured using spectrophotometric methods and individual phenolic compounds were identified and quantified using an HPLC method.

In chapter III, we determined the combined and individual effects of wounding and ultraviolet radiation stresses on total and individual phenolics, antioxidant capacity, and PAL activity. Whole and wounded carrots were subjected to different UV lights: UV-A, B, or C under different length of exposures.

In chapter IV, we studied the possible signal secondary molecules involved in the phenolic synthesis induced by wounding and UV radiation stresses. These molecules include jasmonic acid, reactive oxygen species (ROS), and ethylene. Specific elicitors were applied to the stressed carrot tissue to inhibit the specific stress-induced signaling

molecule production or action. We propose two different diagrams which describe the interactions among these secondary signaling molecules in phenolic synthesis induced by wounding and UV radiation stresses.

CHAPTER II

PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF CARROT TISSUE INCREASE WITH WOUNDING INTENSITY

Synopsis

Wounding stress increases total phenolic contents and antioxidant (AOX) capacity of carrot tissue. However, it is unclear how different wounding intensities (A/W; wounded surface area in cm^2 per weight in g) affect the increase in individual phenolic antioxidants. In this part of the study, the effect of different carrot cultivars and duration of storage on total phenolics and AOX capacity were characterized.

In a first experiment, carrots (*Daucus carota* L.) were shredded and stored for 8 d in the dark at 15°C with measurements done periodically. In a second experiment, carrots were cut into slices, pies, and shreds and stored for 4 d in the dark at 15°C before individual and total phenolics, AOX capacity, and phenylalanine ammonia lyase (PAL) activity quantifications.

Results indicated that phenolics, AOX, and PAL activity increased significantly with increasing wounding intensity. In the first experiment, phenolics, AOX, and PAL activity increased during storage for all shredded carrot cultivars with PAL reaching a maximum activity on day 2. The phenolic compounds showed a linear relationship with AOX for all carrot cultivars. In the second experiment, it was determined that different A/W induced phenolic profiles with different AOX capacities. The linear plot between total AOX and total phenolics showed different slopes for different A/W. The slopes corresponded to the increase in the specific AOX (antioxidant activity per unit weight of phenolic compound) and depended on the phenolic profile. The main phenolics identified were chlorogenic acid and its isomers (dicaffeoylquinic acids), ferulic acid, *p*-hydroxybenzoic acid, hydroxybenzoic acid derivative, and isocoumarin.

Several possible secondary wound signaling compounds that induce these phenolic compounds were discussed. These may include ethylene, jasmonic acid and reactive oxygen species.

Introduction

One of the procedures involved in fresh-cut fruits and vegetables processing is cutting the produce into smaller sizes. This cutting action essentially is causing the tissue to endure wounding stress. Wounding will cause some physiological effects to the tissue and it is understood that the more severe the cutting process, the greater the wounding response will be. Most of these effects are known to decrease the quality of the produce. Wounding has been known to increase respiration rate (Surjadinata and Cisneros-Zevallos 2003) and ethylene production of many different tissues (Rolle and Chism 1987, Saltveit 1997). Many responses occur because wounding stress involves in the generation, translocation, perception, and transduction of signals in order to activate the expression of wound-inducible genes (Leon and others 2001), either locally by the injured cells or systemically by the adjacent cells.

Plant cells are contained inside rigid walls, making each cell to be capable of triggering the defense mechanism. When wounding stress occurs, the cell can activate the specific transcriptional genes (Saltveit 2000, Leon and others 2001), with the purpose of adjusting the metabolism to repair and heal the damage and to synthesize substances to prevent invasion by the predators. These processes could occur between a few minutes to several hours after wounding (Leon and others 2001).

In plants, many compounds have been proposed to play a role in wound signaling, such as phytohormones (ethylene, jasmonic acid, ABA), mitogen-activated protein kinases (MAPK), reactive oxygen species (ROS), salicylic acid, systemin, oligosaccharides, as well as electrical pulses and hydraulic waves (Saltveit 2000, Leon and others 2001, Rakwal and Agrawal 2003). To date, it has not been possible to identify and define which one is the primary signal that triggers the wound-defense mechanism. Whatever the signal may be, an improved knowledge of this signaling mechanism would

be very important in order to understand the effect of this stress on the tissue and to develop strategies to avoid it or enhance it.

A stress or an injury to a plant cell will trigger two types of responses in phenolic metabolism (Rhodes and Wooltorton 1978). The first response is the oxidation of the existing phenolic compounds as a result of rupture of the cell membrane, causing the phenolics to combine with the oxidative enzyme systems. The other response involves the synthesis of monomeric or polymeric phenolics to repair the wounding damage. This second response are caused by changes in phenylalanine ammonia lyase activity (PAL, EC 4.3.1.5) since it is the key metabolic enzyme in the phenylpropanoid pathway (Babic and others 1993, Kang and Saltveit 2002). Increase in phenolic content is one of the most studied phenomena in response to wounding (Toivonen and DeEll 2002). Babic and others (1993) found that storing shredded carrots in air accumulated chlorogenic acid. Leja and others (1997) found that the peels of the carrot slices stored for 4 days at 20^{o}C produced more chlorogenic acid and isocoumarin. Heredia and Cisneros-Zevallos (2002) found that wounding stress increased total phenolic content and therefore increased the AOX capacity of carrot tissue. These findings were also shown in other tissues, such as lettuce leaf (Kang and Saltveit 2002) and purple-flesh potato (Reyes and Cisneros-Zevallos 2003). However, all of these studies were done with only one type of cuts (e.g. slices).

It is important to quantify the change in phenolic content and antioxidant capacity when different wounding intensities are applied. Other factors that need to be considered are duration of storage since it has been shown to be influential (Leja and others 1997) and tissue cultivars since phenolic content can vary greatly between the varieties (Talcott and Horward 1999). Therefore, the objective of this study was to determine the effect of wounding intensity, storage, and cultivars on total and individual phenolic content, AOX capacity, and PAL activity of wounded carrot tissue.

Materials and Methods

Plant materials and reagents

Whole-topped carrots c.v. Navajo, Legend and Choctaw were used in this study. All three cultivars came from California (Grimmway Farms, Bakersfield, CA) and they are commonly used for processing. They were grown under similar conditions and all were harvested within two weeks. These whole carrots were stored in a 2-5^{o}C room until used (within two weeks period). Carrots were conditioned overnight in a 15^{o}C room before the wounding studies. All carrots used in the experiment were approximately the same size (2.5-3 cm in diameter and 22-25 cm in length) to eliminate weight and maturity factors that might influence the total phenolic content and the antioxidant capacity. The carrots were also free of visual damage since any injury prior to processing could induce the phenolic metabolism.

All chemicals and standards used: Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpyrrolidine (PVPP), sodium hydroxide (NaOH), boric acid, 2-mercaptoethanol, chlorogenic acid, *p*-hydroxybenzoic acid, and ferulic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1,3-dicaffeoylquinic acid (cynarin) was purchased from ChromaDex, Inc. (Santa Ana, CA, USA). 1-aminocyclopropene-1-carboxylic acid (ACC) was purchased from MP Biomedicals (Aurora, OH, USA). Methanol, ethanol, hexane, and acetonitrile were reagents of HPLC grade.

Wounding stress

On the day of processing, carrots were taken from the 15^{o}C storage, washed with 100 ppm chlorinated water and air-dried for 2-3 h at room temperature. For the first experiment, carrots were shredded (considered as extreme wounding) using carrot cylinders of 150 g and replicated 6 times. The shreds were obtained using a food processor (High Performance model, West Band Co., West Band, WI). Carrot shreds were stored for 0, 2, 4, and 8 d and measurements of total phenolics, antioxidant capacity, and phenylalanine ammonia lyase (PAL) activity were performed. For the

second experiment, carrots were cut into different wounding intensities (e.g. slices, pies and shreds), while non-wounded carrots were used as the control. Same measurements as explained above were done after 4 d of storage at 15^{o}C.

After cutting, wounded and non-wounded carrots were placed in 4 L closed clear glass jars. The jars were stored at in the dark at 15^{o}C and were ventilated periodically (every 6-8 h) to maintain aerobic respiration and avoid accumulation of carbon dioxide (> 0.5%).

Intensity of wounding

Wounding intensity (A/W) was defined by the ratio of the new surface area created by wounding in cm^2 over the tissue weight in g (Surjadinata and Cisneros-Zevallos 2003). The average diameter of the carrot cylinders used for the different wounding cuts was 2.5 cm and the average length was 13.5 cm. The wounded surface areas of carrots slices, pies, and shreds were manually measured. The slices were 5 mm thick. The pies were obtained from the carrots slices and were cut further into quarters. For shreds, the A/W was estimated from 40 pieces of shredded carrots of about 5.1 g, which gave an area of 119.7 cm^2. The calculated wounding intensities (A/W) were 4.2, 6.0, and 23.5 cm^2/g for carrot slices, pies, and shreds, respectively. Whole carrots, as controls, have an A/W of 0.0 cm^2/g since they were not cut, thus no wounded surface area was present.

Total phenolics

Phenolic content was analyzed using the procedure of Swain and Hillis (1959) with some modifications. This assay uses Folin-Ciocalteu reagent (FCR) to measure a sample's reducing capacity (Huang and others 2005).The exact chemical nature of FCR is unknown however it is believed to contain heterolopyphosphotunstates-molybdates. Sequences of reversible one or two electrons reduction reactions lead to blue solution (Huang and others 2005). Therefore, the total phenolic content is higher as the blue color becomes darker. Phenolic compounds will only react with FCR under basic conditions, thus after combining FCR with the sample, sodium carbonate is added.

Five grams of fresh tissue samples were mixed with 25 mL of methanol and homogenized until reaching uniform consistency using an Ultra Turrax (T 25 basic, IKA Labortechnik, Staufen, Germany). Extracts were stored in covered plastic tubes overnight at 4^{o}C and then centrifuged at 30,000 *g* for 20 min (Beckmann Coulter J2-21, Fullerton, CA). The centrifuge was equipped with a rotor (Beckman Coulter JA-17, Fullerton, CA). A 150 µL sample of the clear supernatant was collected and diluted with 2400 µL of nanopure water. At the same time, a blank (150 µL of nanopure water) was prepared by following the same procedure as the sample. To each diluted sample and blank, 150 µL of 0.25 N Folin-Ciocalteau reagent was added, vortexed and allowed to react at room temperature for 3 min, followed by the addition of 300 µL of 1 N Na_2CO_3. This mixture was allowed to react at room temperature in the dark for 2 h. Readings were done at 725 nm using a photodiode array spectrophotometer (Hewlett-Packard 8425A, Waldbronn, Germany), which was previously blanked with methanol. Total phenolics were expressed as mg chlorogenic acid equivalent/100g fresh weight tissue, based on a developed standard curve.

Individual phenolics

The same extracts used for the total phenolics assay were used for determining the phenolic profiles using an HPLC method of Hale (2003). The samples were run using Waters Millennium 3.2 software (Milford, MA, USA). The HPLC system was equipped with a binary pump system (Waters 515, Milford, MA, USA), an auto-injector (Waters 717 plus, Milford, MA, USA), a photodiode array (PDA) detector (Waters 996, Milford, MA, USA), and a column heater (SpectraPhysics SP8792, San Jose, CA, USA). The column used to separate the phenolic compounds was a 4.6 x 150 mm, 5 µm, C-18 reverse-phase column (Waters Atlantis, Milford, MA, USA), which was maintained at 40^{o}C. The injection volume was 10 µL. Two mobile phases were used: acetonitrile as solvent A and water/HCl adjusted to pH 2.3 as solvent B. The gradient system was 0/85, 5/85, 30/0, 35/0 (min/% solvent A). Both solvents were filtered and degassed before used. Prior to injection, the samples were filtered through a 0.2 µm nylon syringe filter.

Identification and quantification was based on developed standard curves, retention times and UV-Vis spectra.

Dicaffeoylquinic acid

Based on the HPLC method described above, the obtained peaks between 16 and 18 min were collected using a fraction collector (Waters Fraction Collector II, Milford, MA, USA) connected after the PDA detector (Waters 996, Milford, MA, USA). The eluent in each collected fraction was evaporated using a Speed-Vac Concentrator (SC 100, Savant, NY, USA) and then freeze dried (PD-6-54A, FTS Systems, Inc, NY, USA). The accumulated powder was then re-dissolved in ethanol and injected into a time of flight (TOF) mass spectrophotometer equipped with electron spray ionization (ESI) in negative ion mode $(M-H)^-$ (PE Sciex API QStar Pulsar, Concord, Ontario, Canada). The isolated compounds were dissolved in water/methanol (50:50 v/v) acidified with 1% acetic acid. The capillary voltage was 4.5 kV. This mass spectrophotometer procedure was done by the Chemistry Department of Texas A&M University (College Station, TX).

Isocoumarin

Isocoumarin extraction was done by using the procedure of Talcott and Horward (1999) with some modifications. Ten kg of whole carrots were sprayed with 100 ppm of 1-aminocyclopropane-1-carboxylic acid (ACC) and then placed in sealed 4 L glass jars for 4 d in the dark at room temperature. The jars were ventilated every 12 h to avoid CO_2 accumulation (>0.5 %). The carrots were also injected with 1000 ppm of ethylene gas once a day. At the end of storage, carrots were peeled. About 600 g of peels were obtained and these peels were then extracted with 3 L of hexane for 20-24 h. The next day, the liquid phase was partitioned with 1 L of ethanol. The ethanol fraction was divided into ten vials of 1 mL of and injected into an HPLC using the protocol described before. The only peak that appeared around 24 min was confirmed to be isocoumarin by monitoring the λ_{max} of the PDA spectra (absorbance at 267 and 302 nm). In order to

accumulate significant amount of isocoumarin, the injection volume used was 125 µL. Each vial was injected three times. The total fractions collected were around 30 mL and these were evaporated with Speed-Vac Concentrator (SC 100, Savant, NY, USA) and further freeze-dried (Kinetics, NY, USA). The resulting powder was then re-dissolved in 1 mL of ethanol. To quantify the amount of isocoumarin present in the dried powder, the absorbencies at 267 and 302 nm of this ethanolic sample were measured with photodiode array spectrophotometer (Hewlett-Packard 8425A, Waldbronn, Germany). The ratio between the two absorbancies was around 2.25-2.29, which is very similar to the pure isocoumarin (ratio = 2.47) (Sondheimer 1957). Quantification of isocoumarin collected was performed using a molar extinction coefficient in ethanol (ε = 14,800/M cm) mentioned by Sondheimer (1957). In 1 mL of ethanolic samples there was 1.1031 mg of isocoumarin. Several dilutions of this ethanolic sample were injected back into the HPLC to create a standard curve. This time the injection volume was set to be 10 µL, which was the volume injection condition used for the wounded and non-wounded carrot extracts.

Isocoumarin powder collected from the freeze-drier was also re-dissolved in ethanol and injected into a time of flight (TOF) mass spectrophotometer (PE Sciex API QStar Pulsar, Concord, Ontario, Canada) equipped with ESI in positive ion mode $(M+H)^+$ combined with $(M+Li)^+$ for further identification and conformation. The isolated compounds were dissolved in water/methanol acidified with 1% acetic acid. The capillary voltage was 4.5 kV. The mass spectrophotometer procedure was done by the Chemistry Department of Texas A&M University (College Station, TX).

Antioxidant capacity

AOX capacity was quantified using the procedure of Brand-Williams and others (1995). The same extracts prepared for the total phenolics assay were also used for this AOX assay. A diluted solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was prepared from the mother stock solution using methanol to obtain an absorbance of 1.1 ± 0.02. After centrifugation of the extracts at 30,000 *g* (Beckmann Coulter J2-21 equipped with

a rotor JA-17, Fullerton, CA) at 4°C for 20 min, 150 µL of the clear supernatant was mixed with 2850 µL of the diluted DPPH solution in a clean plastic vial, vortexed and then closed. At the same time, a blank of 150 µL of methanol mixed with the DPPH diluted solution was prepared. The sample extract and DPPH was allowed to react in the dark at room temperature for about 24 h or until reaching steady state. This steady state is shown by change in color from dark purple to light purple, which happens when the electrons of DPPH radicals becomes paired with a hydrogen atom from a free radical scavenging antioxidant to form the reduced DPPH-H (Prakash 2001). The resulting decoloration is stoichiometric with respect to the number of electrons captured (Prakash 2001). AOX capacity was measured at 515 nm using a photodiode array spectrophotometer (Hewlett-Packard 8425A, Waldbronn, Germany), that was previously blanked with methanol/DPPH solution. AOX capacity was expressed as µg Trolox equivalents/g fresh weight tissue, based on a developed standard curve using trolox.

PAL activity

PAL activity was measured using the procedure of Ke and Saltveit (1986) with slight modifications. For each replicate, 1 g of tissue was mixed with 0.2 g polyvinylpyrrolidine (PVPP) and homogenized in 30 mL of 50 mM cold borate buffer (pH 8.5) at low speed to a uniform consistency. The borate buffer was a mixture of boric acid and sodium hydroxide in nanopure water containing 400 µL of 2-mercaptoethanol per 1 L buffer. The extracts were then filtered through 4 layers of cheesecloth and centrifuged at 30,000 *g* (Beckmann Coulter J2-21 equipped with a rotor JA-17, Fullerton, CA) for 15 min at 4°C. Throughout the analysis, sample extracts were kept in ice and in dark condition. After centrifugation, clear supernatant was collected and incubated for 5 min in a 40°C water bath. Two sets of 10 mL glass tubes were prepared. The first set is for the water control and the second set is for the samples to which 100 mM of L-phenylalanine was added as substrate. Measurements of the enzyme activity were done at 0 and 1 h of incubation in a 40°C water bath. PAL activity was quantified

as μmoles of *t*-cinnamic acid/h g fresh weight tissue. It was calculated using a standard curve developed for *t*-cinnamic acid in the borate buffer.

Statistical analysis

Statistics analysis was done using the ANOVA procedure from the SAS Statistical Analysis System for Windows v8.1 software (SAS Institute Inc, Cary, NC, USA). The treatment means were compared with Tukey's Studentized Range test at α=0.05.

Results and Discussion

Effect of wounding on total phenolics, PAL activity and AOX capacity during storage

Wounding stress (shredding) induced the accumulation of phenolic compounds in carrot tissue. There was significant increase ($P<0.05$) of total phenolics through storage time. However, there was no significant difference ($P>0.05$) between the carrot cultivars studied. Compared to day 0, the total phenolics increased up to approximately 574%, reaching a maximum after 8 d of storage (Figure 1A).

Results showed that wounding stress activated the phenylpropanoid metabolism by eliciting the synthesis of the pathway key enzyme, PAL. PAL activity increased for all three carrot cultivars reaching a maximum peak after 2 d storage and afterwards decreased through time (Figure 1C). The increase in PAL activity on day 2 was approximately 719%, 751%, and 1172% for Navajo, Legend, and Choctaw cultivar, respectively. The decrease in PAL activity after 48 h may be related to a cell regulatory mechanism involving possibly a PAL-inactivating system (Zucker 1968, Chalutz 1973, Creasy and others 1986). Specific enzyme turnover systems exist and their existence in the tissue is regulated by mechanism similar to those controlling the synthesis of inducible enzymes (Creasy and others 1986, Zucker 1968). Similar pattern of PAL activity elicited by wounding have been described previously for carrot tissue (Babic and others 1993, Ke and Saltveit 1989).

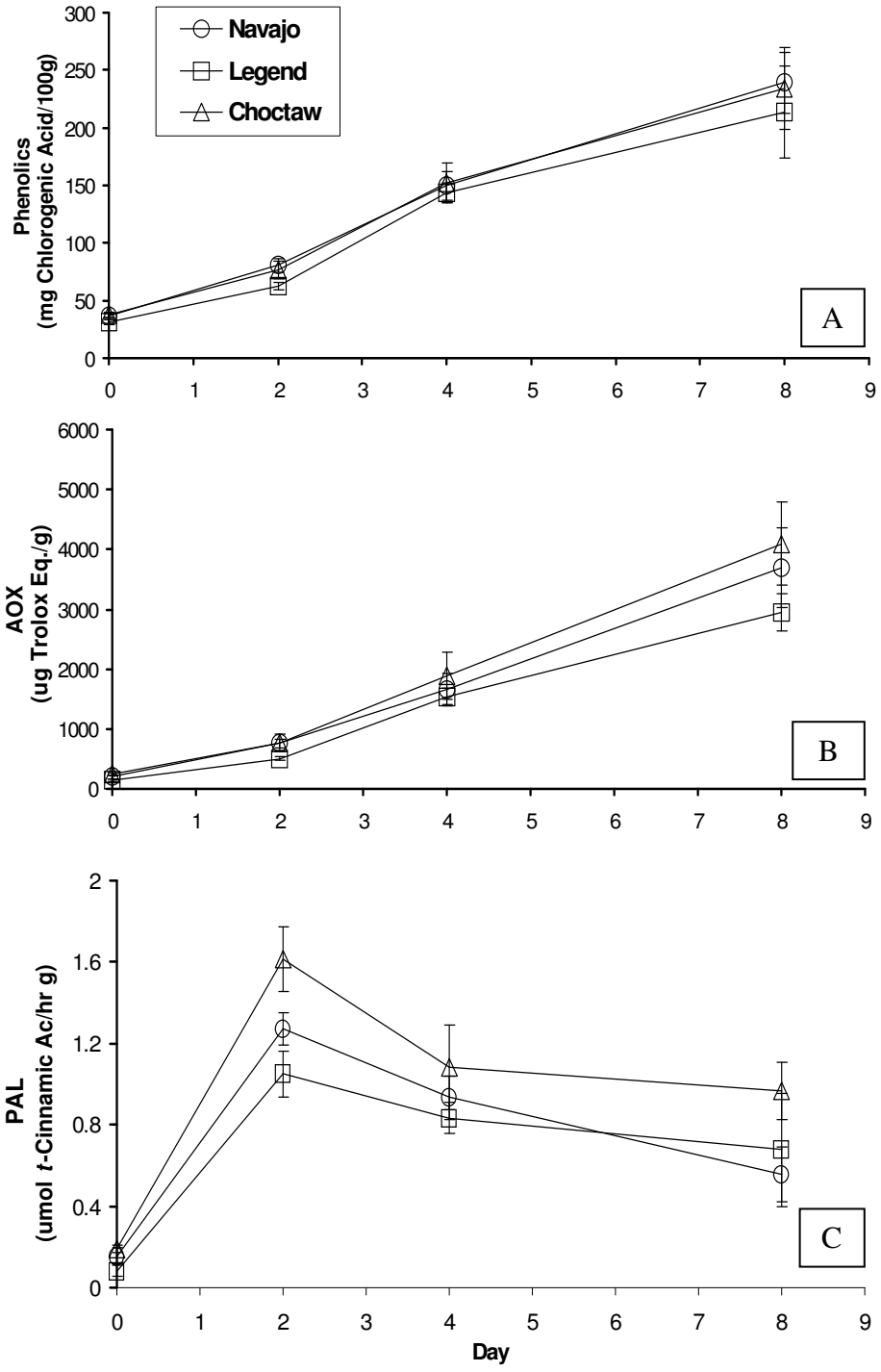

Figure 1 – Total phenolic content (A), antioxidant capacity (B), and PAL activity (C) of different cultivars of shredded carrots during storage at 15°C. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 6).

Total phenolics in wounded carrots continued to accumulate after 4 and 8 d storage since the PAL-synthesizing system remained present. Even though its activity decreased, the enzyme was not completely inactivated, thus maintaining the ability to produce phenolic compounds. Between days 4 and 8 of storage, there was a decline in the rate of synthesized phenolics (Figure 1A).

There was a significant increase ($P<0.05$) in AOX capacity with storage time for all wounded carrot cultivars studied. AOX capacity increased after 8 d storage approximately 1554%, 1859%, and 1700% for Navajo, Legend, and Choctaw cultivar, respectively, compared to day 0 (Figure 1B). This increase in AOX capacity is related to the increase in phenolic compounds obtained in the same storage period. Total phenolics showed a linear relationship with the AOX capacity for each sampling day of storage (Figure 2). This pattern was similar for all cultivars and the linear relationship showed a trend to pass through 0 for both X and Y axes. The slopes of these linear relationships increased as the storage time increased. The slopes correspond to the specific phenolic antioxidant capacity, which is the antioxidant capacity expressed on a phenolic basis (ratio between total AOX and total phenolics) (Table 1). The specific AOX capacity gives information of the effectiveness of the phenolic present on neutralizing the free radicals. Thus, a higher specific AOX (a higher slope) would mean that the phenolics present stabilize a greater number of free radicals. The phenolic which has more antioxidants has higher slope. According to this, the specific AOX capacity for all three cultivars increased approximately 1.6, 2.0, and 2.8 fold after 2, 4, and 8 d of storage, respectively.

After 8 d of storage, water loss of the shredded carrots was approximately 3%. At day 8, wounded carrots showed development of off-odor, possibly due to a combined effect of increase respiration, ethylene production and membrane deterioration at 15^{o}C. This temperature was used in all experiments only to accelerate the wounding response and it is not recommended for maintaining quality. Lower temperature would be more appropriate.

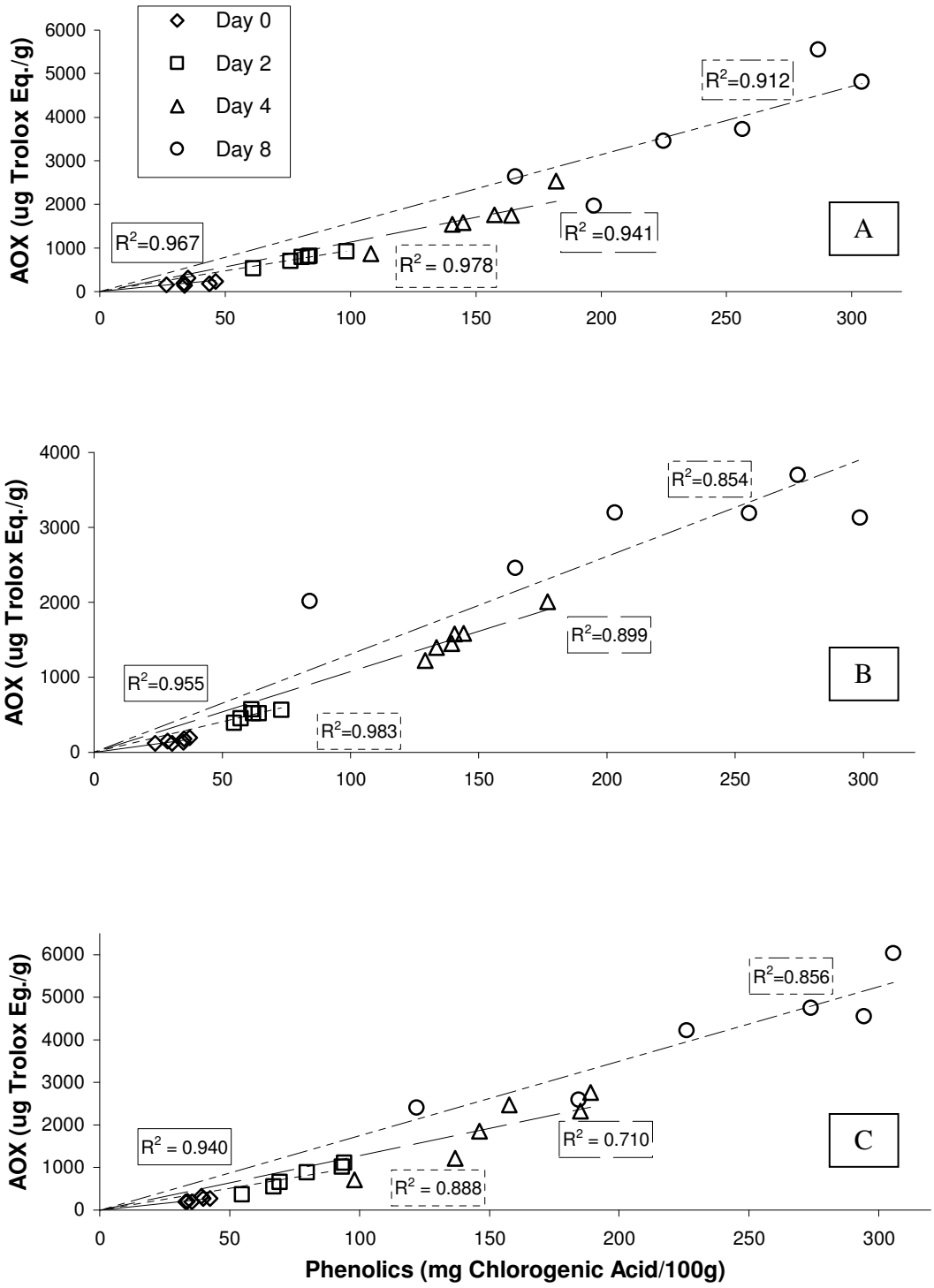

Figure 2 – Relationship between antioxidant capacity and total phenolic content are linear for all storage days at 15°C for c.v. Navajo (A), Legend (B), and Choctaw (C).

Table 1 – Specific antioxidant capacity during storage at 15°C for different carrot cultivars.

Cultivar	Storage Time (d)	Specific AOX* (μg Trolox Eq./mg Phenolics)**
Navajo	0	542 ± 174.39
	2	941 ± 40.46
	4	1116 ± 189.82
	8	1543 ± 303.08
Legend	0	476 ± 69.77
	2	810 ± 72.04
	4	1071 ± 68.80
	8	1382 ± 303.08
Choctaw	0	664 ± 99.54
	2	1002 ± 192.25
	4	1243 ± 327.42
	8	1748 ± 233.58

* Specific activity corresponds to the slopes of the linear regression lines in Figure 2.
** Phenolic content was quantified based on chlorogenic acid (5-CQA) standard.

The standard practice of whole carrot postharvest handling includes storing the clean and fresh carrots in a room of 0-5°C with 98-100% relative humidity (Rubatzky and others 1999). With this storage, the shelf-life of the carrots will be approximately 2 weeks (Rubatzky and others 1999). However, if the carrots are placed in modified atmosphere packaging (MAP) at 0°C and high relative humidity (at least 98%), then the carrots can last up to 1 month (Rubatzky and others 1999). For the remaining experiments, we decided to only store carrot samples up to 4 d at 15°C.

Effect of wounding intensity on total phenolics, PAL activity and phenolic profiles

Results indicated that accumulation of phenolic compounds increased with wounding intensities for all three carrot cultivars studied (Figure 3A). Total phenolics content increased approximately 97%, 76%, and 252% for carrot slices, pies, and shreds, respectively, after 4 d of storage at 15^{o}C. Cultivar Legend showed lower accumulation of phenolic compounds compared to cultivars Choctaw and Navajo at wounding intensity ≥ 6.0 cm^2/g (pies).

Increased wounding intensity also induced an increased in PAL activity among the tree cultivars studied (Figure 3C). PAL activity increased significantly ($P<0.05$) by 1927%, 2790%, and 26624% for carrot slices, pies, and shreds, respectively, compared to non-wounded carrots. Among the carrot cultivars, Navajo and Legend showed very similar PAL activity ($P>0.05$), however for Choctaw cultivar PAL was significantly higher ($P<0.05$) only for shreds. Ke and Saltveit (1989) previously found that PAL activity increased with number of punctures on lettuce tissue. Reported experimental data related to increase in PAL with the increase of wounding is scarce in the literature.

HPLC analysis of the phenolic profiles for wounded and non-wounded carrot tissue showed the same type of phenolic compounds for all three cultivars (Figures 4 to 6 and Table 2). HPLC analysis of phenolic of non-wounded tissue showed the presence of chlorogenic acid or 5-CQA (peak 1) and a dicaffeoylquinic acid isomer, or diCQA (peak 5). As wounding intensity increased, more peaks appeared. Phenolic profiles of carrot slices and pies, showed an additional presence of ferulic acid (peak 3) and isocoumarin (peak 7), while phenolic profile of shredded carrots showed, in addition, peaks for *p*-hydroxybenzoic acid or *p*HBA (peak 2), an unknown hydroxybenzoic acid derivative (peak 6), and one more diCQA (peak 4). Peak 6 had very similar PDA spectra to *p*-hydroxybenzoic acid (Figures 7 and 8), however the retention times differed by about 18 min, meaning that this compound is more non-polar compared to *p*-hydroxybenzoic acid.

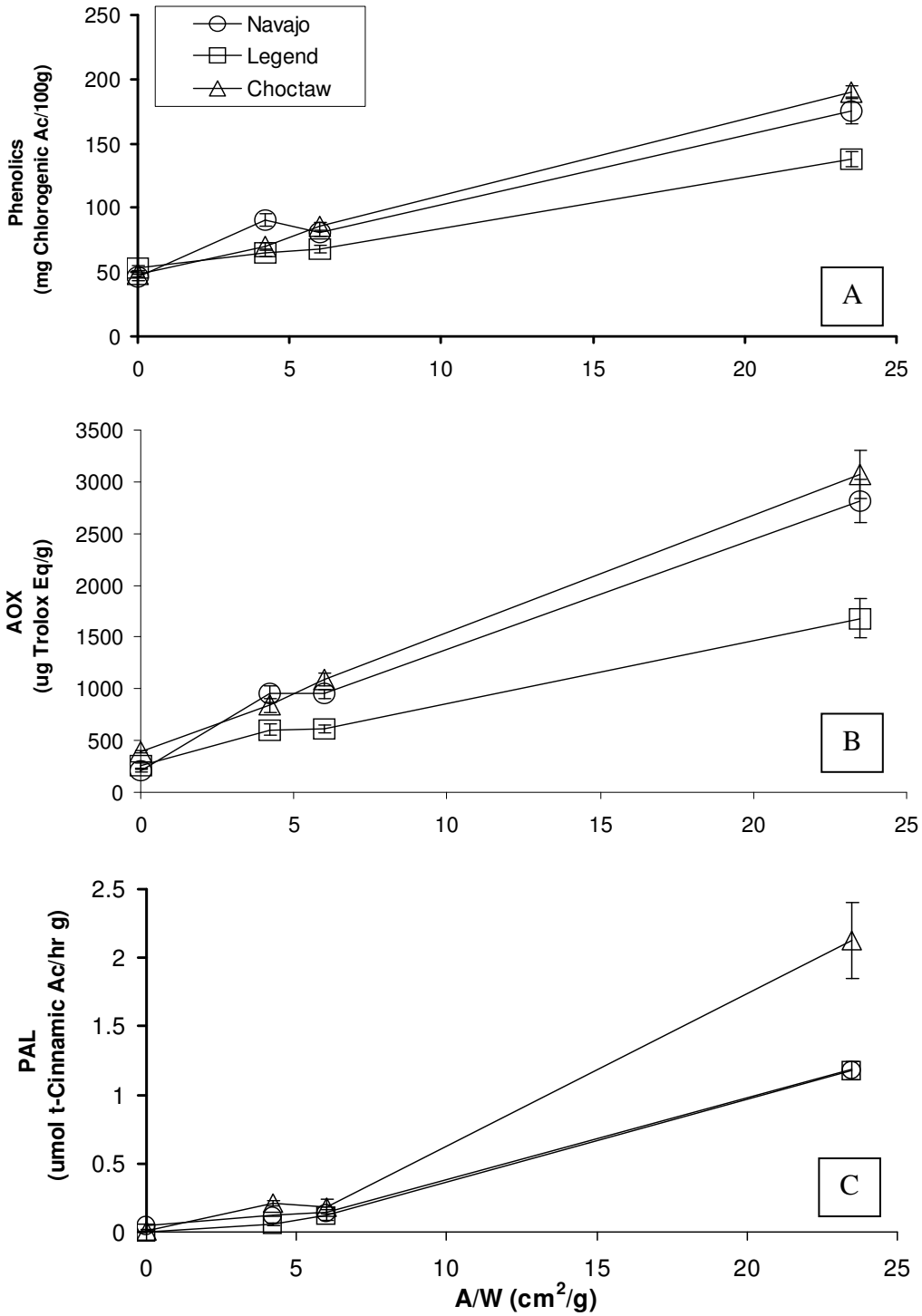

Figure 3 – Total phenolic content (A), antioxidant capacity (B), and PAL activity (C) of the different wounding intensity (A/W) and carrot cultivars after 4 d storage at 15°C. Vertical bars represent SD (*n*=6).

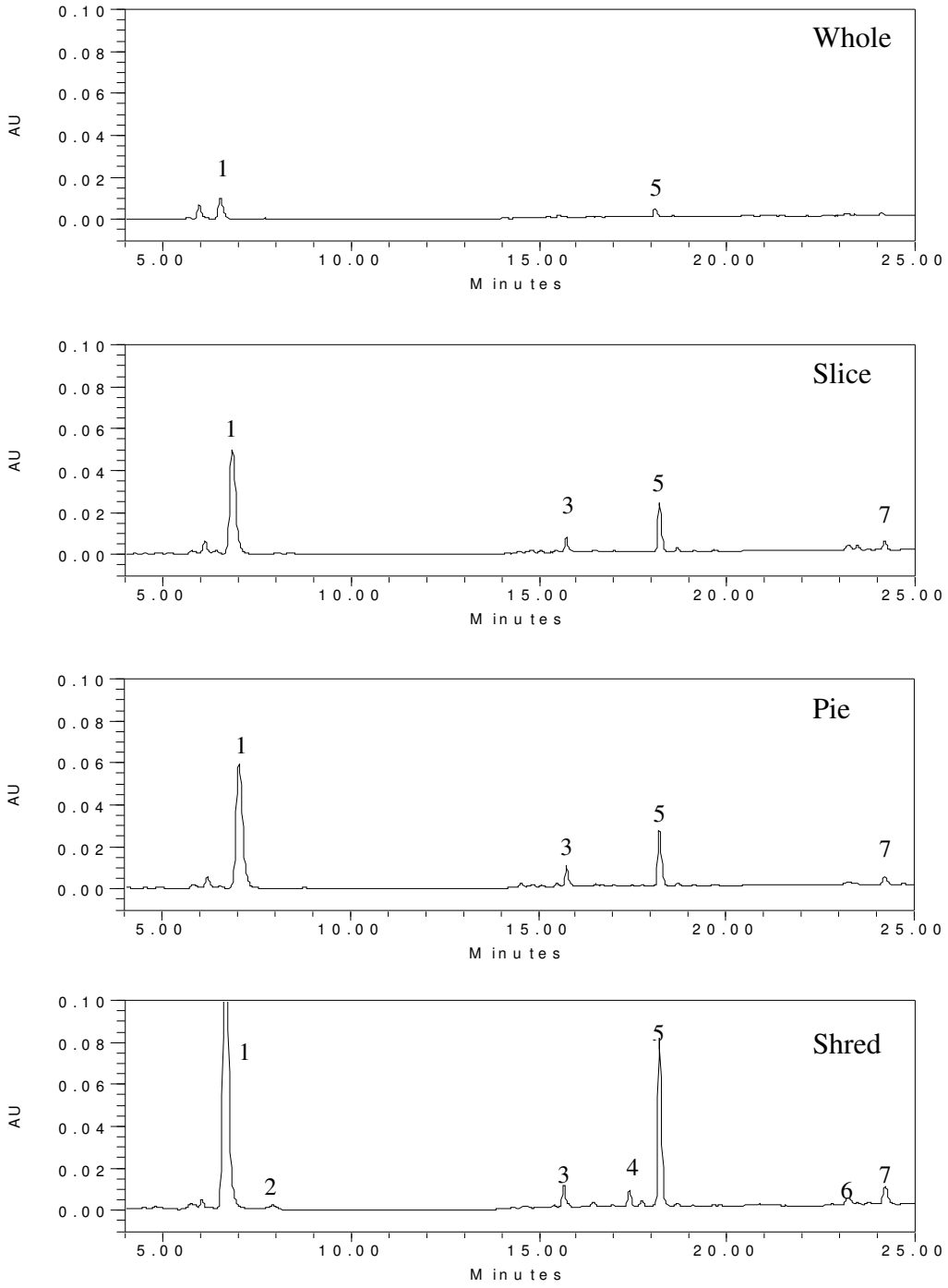

Figure 4 – HPLC phenolic profiles of carrot tissue at 280 nm for c.v. Navajo. Peaks: 1 = chlorogenic acid (5-CQA), 2 = *p*-hydroxybenzoic acid (*p*HBA), 3 = ferulic acid (FA), 4 = 3,4-dicaffeoylquinic acid (3,4-diCQA), 5 = 3,5-dicaffeoylquinic acid (3,5-diCQA), 6 = hydroxybenzoic acid derivative, 7 = isocoumarin. AU = absorbance unit. X-axis is retention time in min.

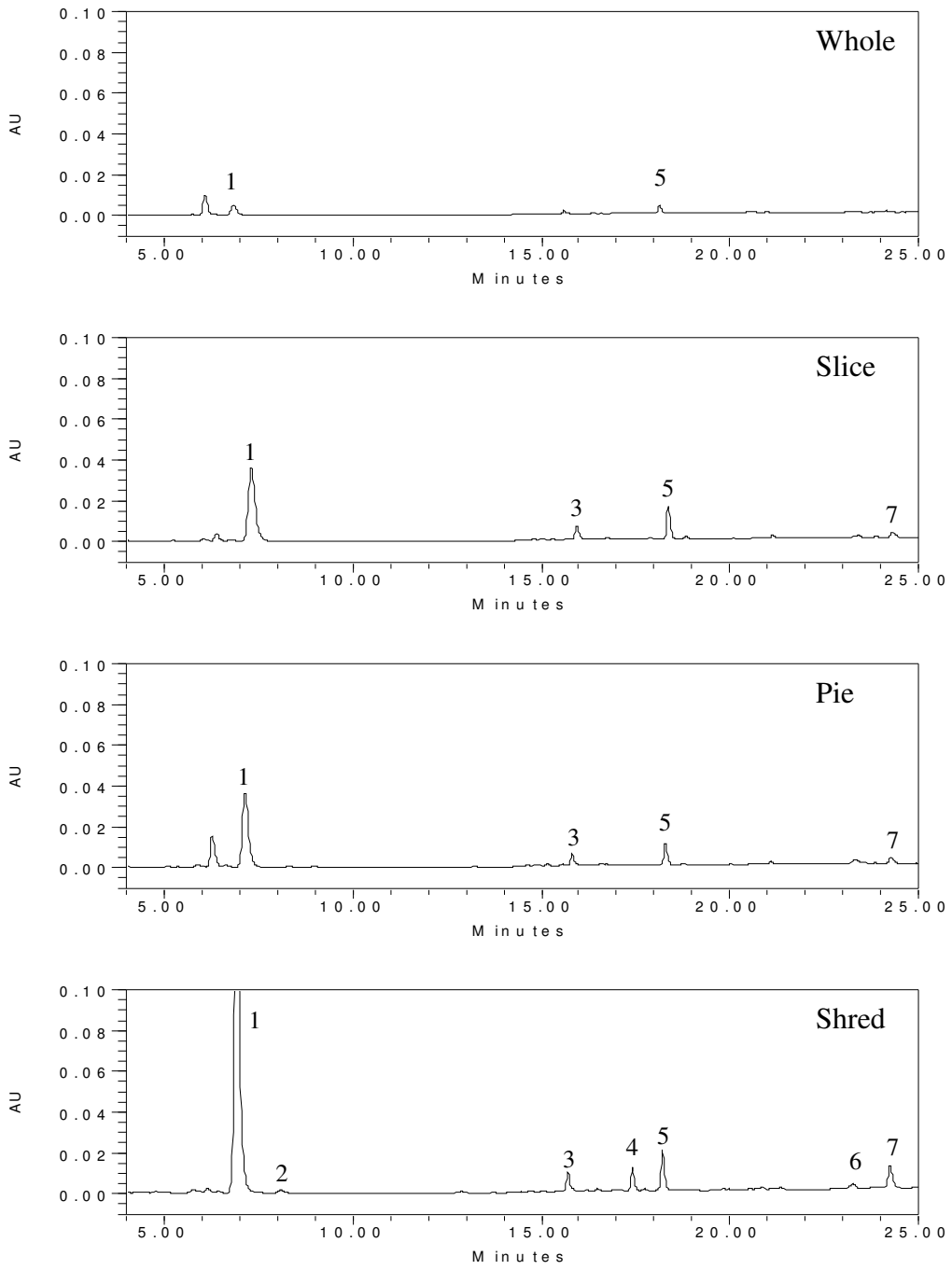

Figure 5 – HPLC phenolic profiles of carrot tissue at 280 nm for c.v. Legend. Peaks: 1 = chlorogenic acid (5-CQA), 2 = *p*-hydroxybenzoic acid (*p*HBA), 3 = ferulic acid (FA), 4 = 3,4-dicaffeoylquinic acid (3,4-diCQA), 5 = 3,5-dicaffeoylquinic acid (3,5-diCQA), 6 = hydroxybenzoic acid derivative, 7 = isocoumarin. AU = absorbance unit. X-axis is retention time in min.

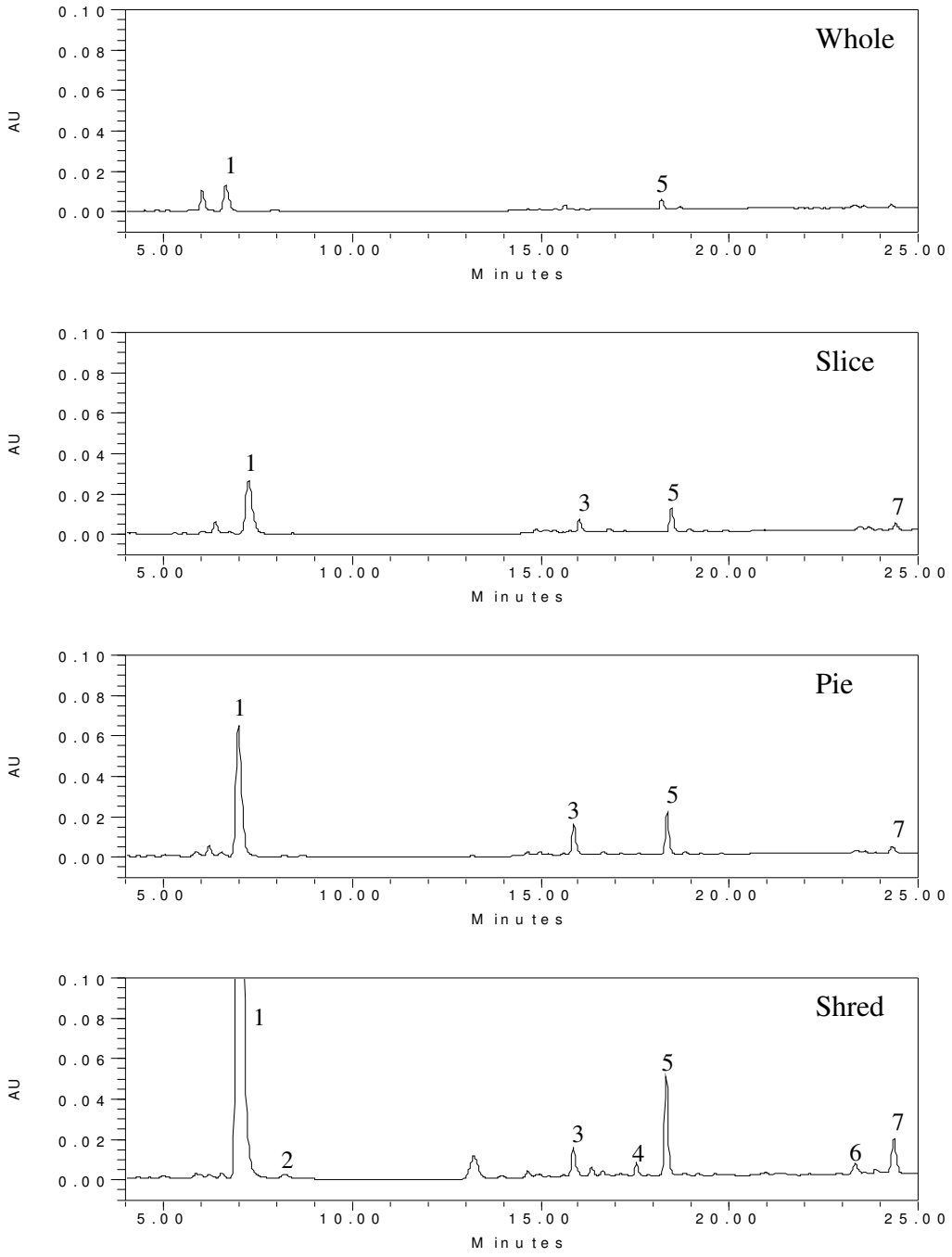

Figure 6 – HPLC phenolic profiles of carrot tissue at 280 nm for c.v. Choctaw. Peaks: 1 = chlorogenic acid (5-CQA), 2 = *p*-hydroxybenzoic acid (*p*HBA), 3 = ferulic acid (FA), 4 = 3,4-dicaffeoylquinic acid (3,4-diCQA), 5 = 3,5-dicaffeoylquinic acid (3,5-diCQA), 6 = hydroxybenzoic acid derivative, 7 = isocoumarin. AU = absorbance unit. X-axis is retention time in min.

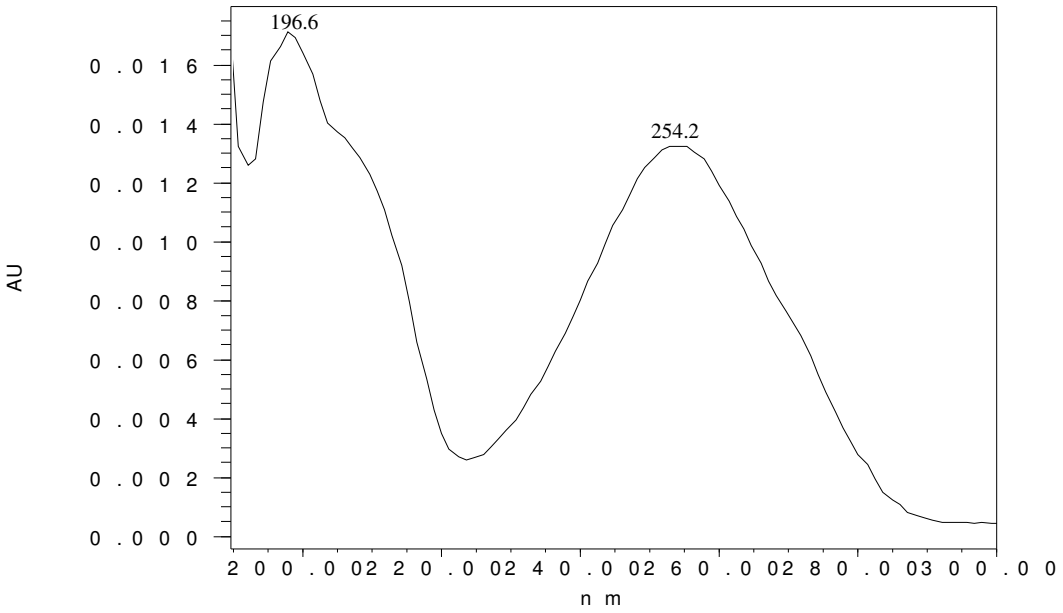

Figure 7 - Photodiode array (PDA) spectra of peak 2 = *p*-hydroxybenzoic acid (*p*-HBA) identified by HPLC at 280 nm. Retention time = 7.92 min. AU =absorbance unit.

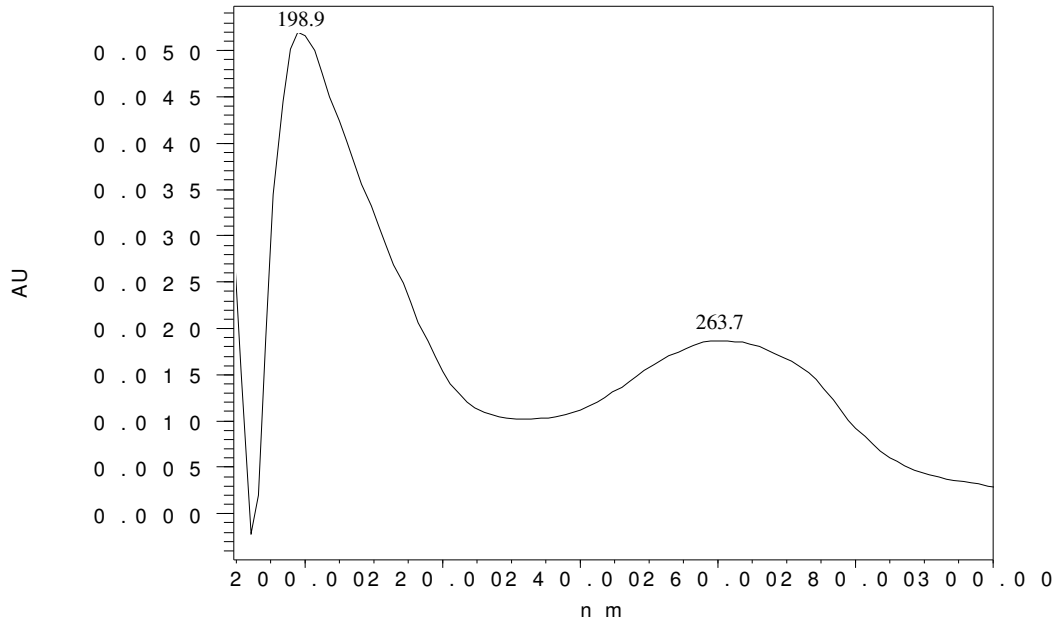

Figure 8 - Photodiode array (PDA) spectra of peak 7 = hydroxybenzoic acid derivative (HBA) identified by HPLC at 280 nm. Retention time = 23.19 min. AU = absorbance unit.

Table 2 – Phenolic contents of different cultivars and wounding intensity, quantified by HPLC at 280 nm.

Cultivar	Peak #	Ret Time (min)	λmax (nm)	Compound	Amount (mg/100g)			
					Whole	Slices	Pies	Shreds
Navajo	1	6.85	193.1, 216.5, 241.3, 325.5	5-CQA	5.499	15.71	22.34	52.54
	2	7.92	196.6, 254.2	*p*-HBA	nd*	nd	nd	0.819
	3	15.57	195.4, 216.5, 234.2, 321.9	FA	nd	2.099	2.200	2.740
	4	17.47	196.6, 241.3, 326.7	3,4-diCQA**	nd	nd	nd	1.399
	5	18.15	197.8, 271.7, 240.1, 326.7	3,5-diCQA**	4.593	7.670	8.250	8.214
	6	23.19	198.9, 263.7	HBA Derivative***	nd	nd	nd	1.718
	7	24.19	214.2, 267.1, 302.3	Isocoumarin	nd	0.089	0.095	0.734
				Total Phenolics**	**45.85**	**90.39**	**80.39**	**175.7**
Legend	1	6.85	193.1, 216.5, 241.3, 325.5	5-CQA	5.291	12.64	13.04	38.67
	2	7.92	196.6, 254.2	*p*-HBA	nd	nd	nd	0.157
	3	15.57	195.4, 216.5, 234.2, 321.9	FA	nd	2.073	2.055	2.386
	4	17.47	196.6, 241.3, 326.7	3,4-diCQA	nd	nd	nd	1.656
	5	18.15	197.8, 271.7, 240.1, 326.7	3,5-diCQA	4.472	6.441	6.158	7.887
	6	23.19	198.9, 263.7	HBA Derivative	nd	nd	nd	0.616
	7	24.19	214.2, 267.1, 302.3	Isocoumarin	nd	0.079	0.080	0.301
				Total Phenolics	**52.70**	**64.72**	**68.02**	**137.6**
Choctaw	1	6.85	193.1, 216.5, 241.3, 325.5	5-CQA	6.302	15.44	23.29	71.68
	2	7.92	196.6, 254.2	*p*-HBA	nd	nd	nd	0.639
	3	15.57	195.4, 216.5, 234.2, 321.9	FA	nd	2.080	2.810	3.032
	4	17.47	196.6, 241.3, 326.7	3,4-diCQA	nd	nd	nd	1.363
	5	18.15	197.8, 271.7, 240.1, 326.7	3,5-diCQA	4.840	6.613	7.372	8.677
	6	23.19	198.9, 263.7	HBA Derivative	nd	nd	nd	2.239
	7	24.19	214.2, 267.1, 302.3	Isocoumarin	nd	0.053	0.088	0.580
				Total Phenolics	**48.41**	**70.14**	**85.18**	**189.9**

* nd = not detected
** This compound was quantified using chlorogenic acid (5-CQA) as standard.
*** This compound was quantified using *p*-hydroxybenzoic acid (*p*-HBA) as standard.
**** Total Phenolics were quantified spectrophotometrically based on mg chlorogenic acid equivalent/100g FW.

Peaks 4 and 5 were identified as isomers of chlorogenic acid since the PDA spectra (Figure 10) were similar to that of chlorogenic acid (Figure 9). This was then confirmed by the mass spectrophotometer analysis. The m/z of the parent ion of peaks 4 and 5 were 515.11. According to Clifford and others (2003), the m/z for the parent ion of 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA in coffee bean are 515.7, 515.2, and 515.4, respectively. There are 4 different diCQA: 1,3-diCQA (also known as cynarin), 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA (Clifford 1986, Clifford and others 2003). In this study, commercially available 1,3-diCQA (with m/z of 515.12) was injected in the HPLC system and its retention time was about 14 min (data not shown), differing from peak 4 (17.47 min) and peak 5 (18.15 min).

In carrot tissue, 3,5-diCQA has been shown to be in higher amounts compared to other diCQA isomers (Alasalvar and others 2001, Zhang and Hamauzu 2004). According to this, in our study, peak 5 would correspond to 3,5-diCQA, while peak 4 which appeared before peak 5, would correspond to 3,4-diCQA since this isomer is more polar than 3,5-diCQA.

Results showed that chlorogenic acid was the major phenolic compound present in all three carrot cultivars of wounded and non-wounded tissue (Table 2). Babic and others (1993) and Alasalvar and others (2001) also reported that chlorogenic acid was the main phenolic compound in carrot tissue. When carrots were shredded, this compound showed an approximately 8.4, 7.3, and 11.3 fold increase in content for cultivar Navajo, Legend, and Choctaw, respectively (Table 2).

Peak 7 was confirmed to be isocoumarin by MS spectra analysis. The positive ionization and injection of internal standard, lithium, gave m/z of 215.08. Isocoumarin has a mass of 208.21 and lithium has a mass of 7, therefore when combined, the m/z would be 215. The maximum level of isocoumarin produced by the wounded carrot tissue was approximately 0.301-0.734 mg/100 g fresh weights. Isocoumarin is synthesized by wounding in carrot tissue and plays a major role in plant defense system (Fan and others 2000). This compound gives bitter flavor, which could decrease the quality of the wounded carrots.

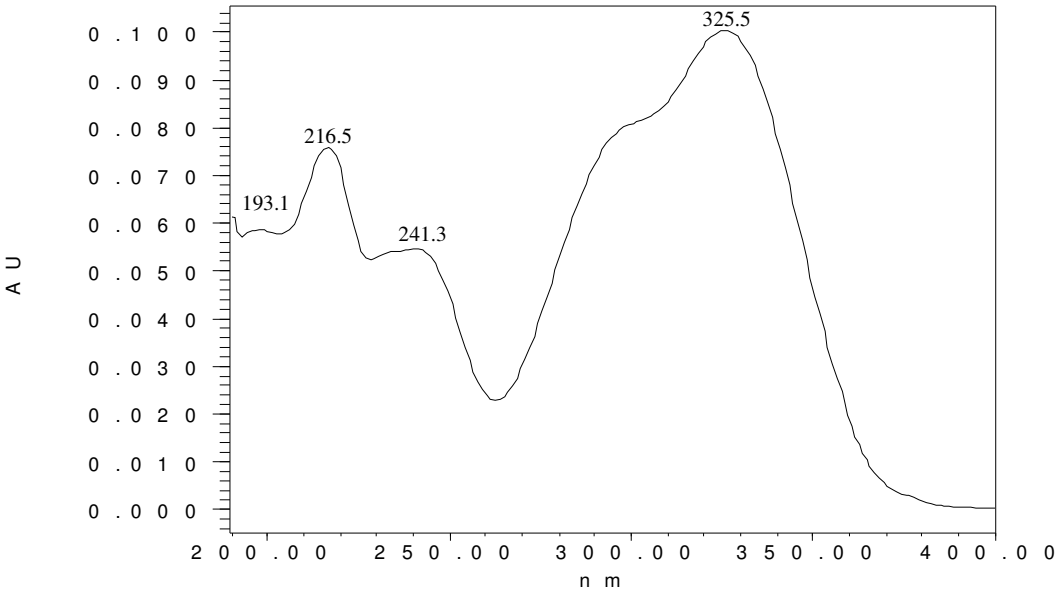

Figure 9 - Photodiode array (PDA) spectra of peak 1 = chlorogenic acid (5-CQA) identified by HPLC at 280 nm. Retention time = 6.85 min. AU = absorbance unit.

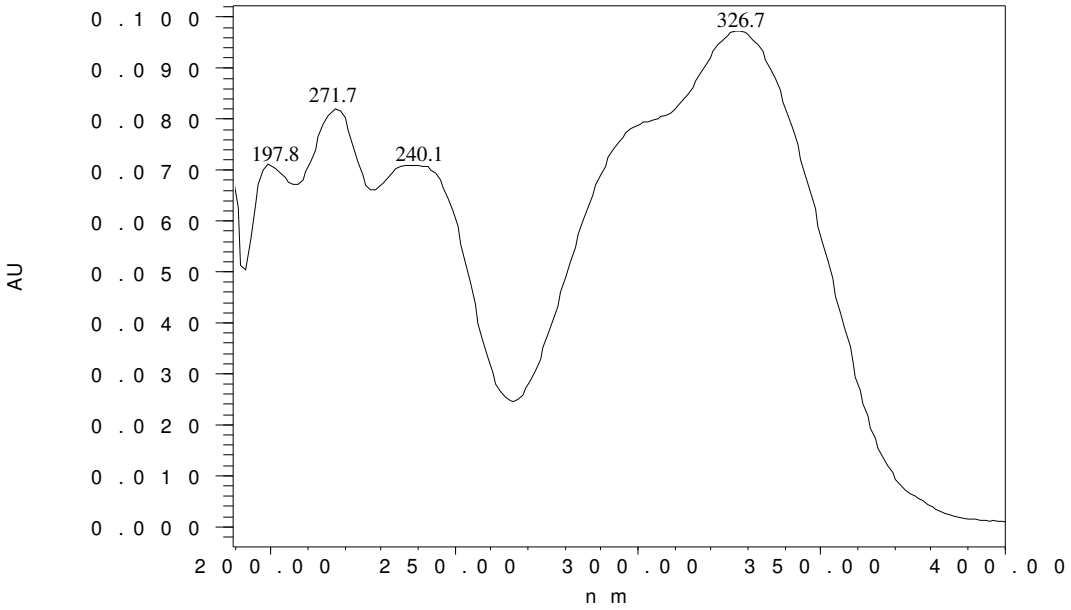

Figure 10 - Photodiode array (PDA) spectra of peak 5 = 3,5-dicaffeoylquinic acid (3,5-diCQA) identified by HPLC at 280 nm. Retention time = 18.15 min. AU = absorbance unit.

According to Lafuente and others (1996), for bitterness flavor to be detected in carrot tissue, the amount of isocoumarin needs to be ≥ 20 mg/100 g of fresh weight. The amount of isocoumarin observed in this study (≤ 1 mg/100 g of fresh weight) will not induce a bitter taste to the tissue. It has been shown previously that other stresses such as ethylene exposure may elicit more isocoumarin synthesis compared to wounding Lafuente and others 1996).

Effect of wounding intensity on AOX capacity

Results indicated that AOX capacity increased with wounding intensity for all three cultivars studied (Figure 3B). The AOX capacity increased by approximately 354% for slices and pies and by approximately 1246% for shreds compared to non-wounded carrot after 4 d of storage at 15^{o}C. AOX capacity was not significantly different ($P>0.05$) between slices and pies for each cultivar. Cultivar Legend showed lower AOX compared to Choctaw and Navajo cultivars for all wounding intensities.

AOX capacity showed a linear relationship with total phenolic contents for all wounding intensities (Figure 11). All three carrot cultivars showed similar trend, where for higher wounding intensities, the slopes of the linear relationship increased and thus, specific AOX capacity increased. The increase in slopes as wounding intensity increased was an indication that wounding stress synthesized phenolic compounds with increased AOX properties. The specific AOX will depend on HPLC phenolic profiles obtained for each wounding intensity (Figures 4 to 6).

Table 3 showed the relative proportions (phenolic profile) of the three major hydroxycinnamic acids present in the three cultivars of wounded and non-wounded carrot tissue: chlorogenic acid (5-CQA), ferulic acid (FA), and one of the chlorogenic acid isomers (3,5-diCQA). Results showed that different types of wounding intensities (A/W) have different relative proportions of phenolic compounds. For non-wounded carrot tissue, there was similar proportion between 5-CQA and 3,5-diCQA, which corresponded to a lower specific AOX capacity. For slices and pies, the phenolic profiles were similar, showing a higher amount of 5-CQA compared to 3,5-diCQA.

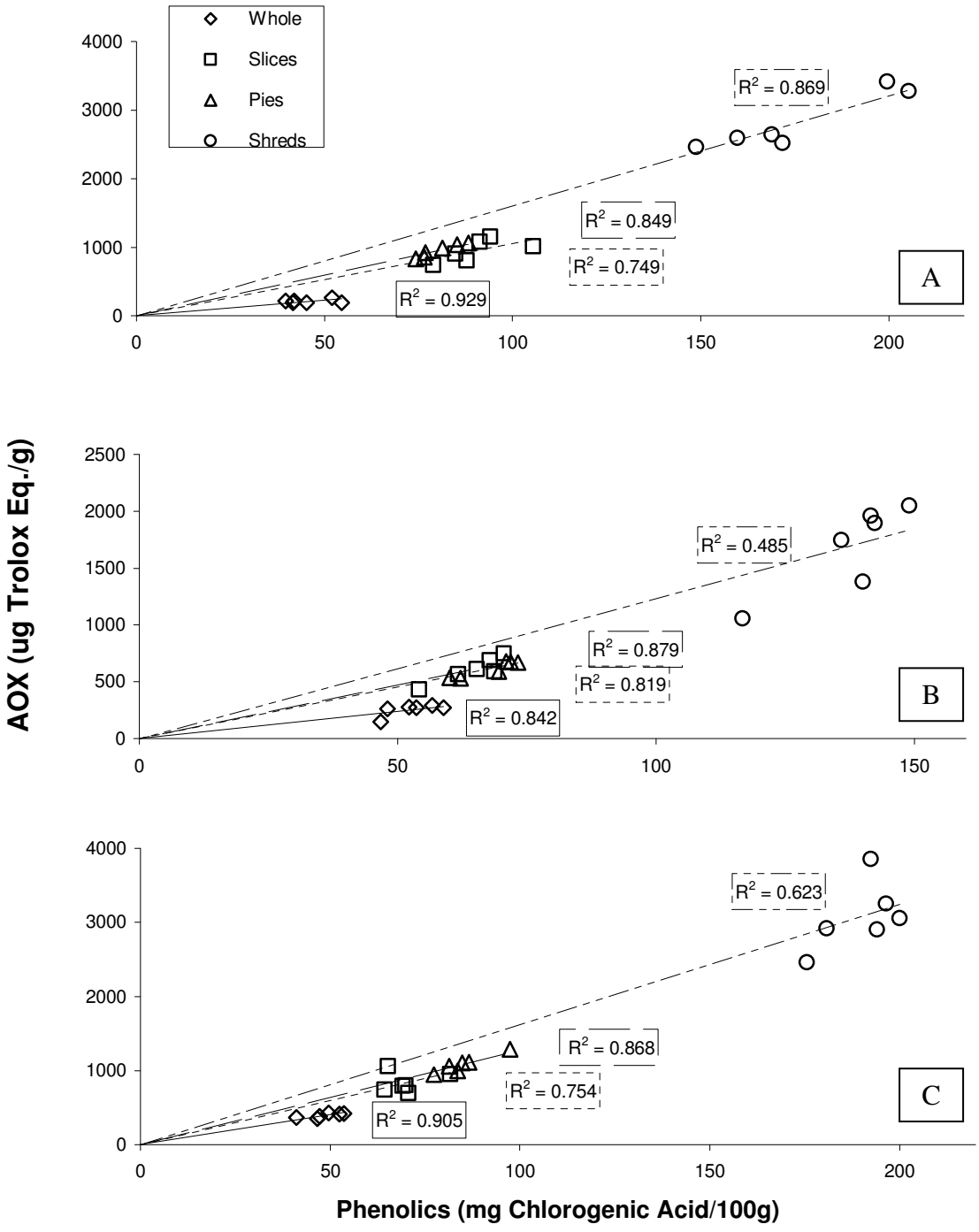

Figure 11 – Relationship between antioxidant capacity and total phenolic content are linear for carrots with different wounding intensities (A/W) for c.v. Navajo (A), Legend (B), and Choctaw (C). Measurements were taken after 4 d storage at 15°C.

These phenolic profiles also give similar specific AOX capacity. This would explain why the slopes of the linear relationship between total AOX capacity and total phenolics were similar for both wounding intensities (Figure 11). For shreds, which showed the highest specific AOX capacity, the relative proportions of phenolic compound indicate a much higher contribution of 5-CQA compared to 3,5-diCQA. These results would suggest that 5-CQA content has a large effect on the specific AOX capacity of carrot tissue.

Table 3 – Relative proportions of the three major hydroxycinnamic acids present (%) and the specific AOX for different A/W.

Cultivar	Cut	A/W (cm^2/g)	5-CQA	FA	3,5-diCQA	Specific AOX (ug Trolox/mg Phenolic)*
Navajo	Whole	0.0	54.5	0.0	45.5	456 ± 71.57
	Slices	4.2	61.7	8.2	30.1	1052 ± 131.82
	Pies	6.0	68.1	6.7	25.2	1180 ± 47.69
	Shreds	23.5	82.7	4.3	12.9	1604 ± 83.51
Legend	Whole	0.0	54.2	0.0	45.8	482 ± 84.24
	Slices	4.2	59.8	9.8	30.4	936 ± 97.21
	Pies	6.0	61.4	9.7	29.0	902 ± 43.40
	Shreds	23.5	79.0	4.9	16.1	1221 ± 211.11
Choctaw	Whole	0.0	56.6	0.0	43.4	809 ± 53.66
	Slices	4.2	64.0	8.6	27.4	1198 ± 213.89
	Pies	6.0	69.6	8.4	22.0	1275 ± 49.90
	Shreds	23.5	86.0	3.6	10.4	1619 ± 210.03

* Phenolic content was quantified based on 5-CQA spectrophotometric standard curve.

Chlorogenic acid has very strong antioxidant activity in carrot (Alasalvar and others 2001). Phenolic antioxidants have a primary function as free radical scavengers and terminators by donating hydrogen atom or electron (Robbins 2003). In general, the more available hydroxyl groups the phenolic has, the more potent it is as antioxidant. Additionally, in order to be a successful antioxidant, the phenoxy radical formed after donating the hydrogen atom, must not produce further radicals but must rather be a relatively stable species (Robards and others 1999).

Potential signaling mechanism of wounding stress in carrot tissue

When a plant is wounded or injured as a response to attack by pathogens or predators, it would activate a variety of defense mechanisms. There have been several proposed signaling mechanisms reported. The main ones are through production of ethylene, jasmonic acid (JA), and reactive oxygen species (ROS) (Saltveit 2000, Leon and others 2001, Zhao and others 2005). These mechanisms may occur directly in the damaged tissue (local response) or in the non-wounded areas (systemic response) (Leon and others 2001).

Ethylene biosynthesis is increased by stress, such as wounding, which triggers the transcription of ACC synthase mRNA (Taiz and Zeiger 1998). This wound-induced ethylene is involved in initial stress responses such as abscission, senescence, wound healing, and disease resistance, which then produces defense-related compounds, such as phenolics (Masia 2003). One might assume that the increase in wound-induced phenolics and PAL activity is caused by wound-induced ethylene production. However, Ke and Saltveit (1989) found that PAL activity induced by wounding appeared much faster than that activated by ethylene. Therefore, the ethylene produced by wounding did not cause the generation of wound-induced PAL activity.

In previous studies, when exogenous ethylene was applied to carrot root, the content of phenolics was significantly increased, especially isocoumarin. A study reported by Fan and others (2000) found that the synthesis of isocoumarin is not due to the amount of ethylene present but rather due to exogenous ethylene action. Application of aminoethoxyvinylglycine (AVG) as ethylene synthesis inhibitor only partially inhibited the accumulation of isocoumarin, while 1-methylcyclopropene (1-MCP) as ethylene action inhibitor was totally repressed isocoumarin synthesis. Sarkar and Phan (1979) mentioned that isocoumarin production was influenced by ethylene and respiration rate of wounded carrots. Therefore, only when wounding induces ethylene, then isocoumarin could be synthesized. Wounding itself is not enough to produce isocoumarin (Lafuente and others 1996). This could explain why the amount of

isocoumarin in the present study was very low in wounded carrots (less than 1 mg/100 g of fresh weight tissue).

Jasmonic acid (JA), through the octadecanoid pathway, is known to be one of the wound-induced secondary signaling molecules (Leon and others 2001). It was reported that JA increased in different plants, only in minutes to several hours after the tissue was wounded (Rakwal and Agrawal 2003) and most of the time, both JA and ethylene are simultaneously required for the activation of wound signaling mechanism (Leon and others 2001). JA and its related compounds have been observed to be one of the signals in the production of plant secondary metabolites (Zhao and others 2003). Endogenous and exogenous JA and methyl jasmonates have shown to induce a wide variety of terpenoids, alkaloids, and phenylpropanoid compounds (Zhao and others 2003). Therefore, the accumulation of phenolic compounds in wounded carrots could be due in part to the production of endogenous JA caused by wounding.

Another common early response of plant cell to stress is oxidative burst (Zhao and others 2005). Wounding can trigger the production of ROS, in part, through the respiration electron transport system. Respiration rate increases as wounding intensity increases and could cause the increase rate production of superoxide (O_2^-) (Purvis 2003). The increase in phenolic compounds in the present study could also be due to the increase in ROS. Both O_2^- and hydrogen peroxide (H_2O_2) have been proposed to function as secondary signals or messengers that increase the production of AOX and ROS scavenging enzymes in tissue of many plants during acclimatization or exposure to mild stress (Purvis 2003), as well as the induction of different plant secondary metabolites, such as furanocoumarin as phytoalexin in parsley cell culture, isoflavanoid glyceollin in soybean, *p*-coumaroyloctopamine in potato, and saponin in ginseng (Zhao and others 2005).

In summary, in this study it was found that both total phenolics and AOX capacity increased, which could suggest that wounded carrot tissue may have enhanced health properties. The mechanism of antioxidant activity of phenolics is believed to be due to donation of hydrogen atom (Robbins 2003). Phenolics act as free radical

scavengers and terminators due to the reactivity of the phenol moiety, which is the hydroxyl substituent on the aromatic ring. The presence of these hydroxyl groups affects the stabilization and the radical-quenching ability of these phenolics (Robards and others 1999, Robbins 2003). Different phenolic compound therefore have different antioxidant activity, for example, hydroxylated cinnamic acids are more potent than benzoic acid compounds and the activity becomes more powerful in caffeic and chlorogenic acids (Hudson and Mahgoub 1980). In carrots, chlorogenic acid (5-CQA) and other hydroxycinnamic acid derivatives are reported to be the main phenolic compounds synthesized after wounding (Babic and others 1993, Alasalvar and others 2001, Zhang and Hamauzu 2004). This would indicate that the increase in specific wound-induced AOX capacity during storage observed in the present study would be most likely associated with the synthesis of phenolic acid group. The HPLC data showed that chlorogenic acid and its related isomers were the major phenolic present and synthesized by wounding. The relative proportions of these groups of phenolic acids were causing the specific AOX capacity to increase since these are very powerful antioxidants. The content of 5-CQA, FA and 3,5-diCQA increased as A/W increased, thus overall AOX capacity of our samples also increased significantly ($P<0.05$) and the specific AOX capacity depended to the relative proportions or HPLC phenolic profiles.

At present, it is still unclear, which of the signaling molecules or combinations of these are responsible for the wound-induced phenolic synthesis and its antioxidant property. It is known that the induction of wound responses requires a simultaneous action of different signals and regulators and participation of any of these signals depends on the species of the plant (Leon and others 2001), therefore further studies are needed to identify and to understand the primary signals that trigger phenolic metabolism.

Conclusions

From the first experiment, it was determined that phenolics and AOX capacity increased during storage for all carrot cultivars of shredded and non-wounded tissue, with the highest increased shown after 8 d storage at 15^{o}C. The increased in phenolic contents was induced by the synthesis of PAL enzyme, which is the key metabolic enzyme of phenylpropanoid pathway. PAL activity reached its maximum after 2 d storage.

On the second experiment, it was determined that different wounding intensity (A/W) induced the synthesis of different phenolic compounds for all carrot cultivars studied of wounded and non-wounded tissue stored for 4 d at 15^{o}C. Increased in wounding intensities also induced an increase in PAL activity. There is linear relationship between total AOX capacity and total phenolics. The increase in slopes as wounding intensity increased was an indication that wounding stress synthesized phenolic compounds with different AOX properties.

The phenolic compounds identified were chlorogenic acid and its isomers (3,4- and 3,5-dicaffeoylquinic acids), ferulic acid, *p*-hydroxybenzoic acid, and isocoumarin. Several possible wound-induced secondary signaling molecules that induce these phenolic compounds are ethylene, jasmonic acid and reactive oxygen species.

CHAPTER III

WOUNDING AND ULTRAVIOLET RADIATION INCREASE ANTIOXIDANT CAPACITY AND CHANGE PHENOLIC PROFILE OF CARROT TISSUE

Synopsis

Previously, we found that phenolic content and antioxidant capacity (AOX) in carrot tissue increased with wounding intensity (A/W). It has also been reported that UV radiation may trigger the phenylpropanoid metabolism in plant tissues. Thus, by selective combination of stresses it may be possible to enhance the content of health promoting antioxidant compounds in plant tissues. The objective of this study was to determine the combined effect of wounding intensity (A/W) and UV-radiation on phenolic compounds, AOX, and the phenylalanine ammonia lyase (PAL) activity of carrots.

Results indicated that phenolic content, AOX, and PAL activity increased in cut carrots with duration of UV-C radiation. Carrot pies showed a higher increase compared to the other cuts. Phenolics, AOX, and PAL activity were also increased when cut carrots were exposed to UV-A or UV-B radiations. The phenolic compounds detected by HPLC include chlorogenic acid and its isomers, ferulic acid, *p*-coumaric acid derivative, *p*-hydroxybenzoic acid and its derivative, and isocoumarin. The type of UV radiation resulted different the phenolic profiles. Chlorogenic acid was induced by all UV radiations, while ferulic acid was induced only by UV-A and UV-B. On the other hand, isocoumarin was induced only by UV-B and UV-C. Other phenolics, such as *p*-hydrobenzoic acid, were not affected by UV radiation. In general, total phenolics were linearly correlated with AOX for all treatments.

There are several proposed signaling mechanisms which induce the synthesis of phenolics caused by UV radiation. Some of them, such as reactive oxygen species, jasmonic acid and ethylene, are the same signaling molecules that could be involved in the phenolic synthesis when the tissue is subjected to wounding stress.

Introduction

In the previous chapter II, we determined that the phenolic content and antioxidant capacity (AOX) in carrots increased with wounding intensity. These changes in phenolic content are caused by the changes in the activity of the phenylalanine ammonia lyase (PAL), the key enzyme in the phenylpropanoid pathway.

Several studies have reported that UV-radiation may trigger the phenylpropanoid metabolism in plant tissues (Arakawa 1988, Dong and others 1995, Jenkins and others 1997, Cantos and others 2001, Reay and Lancaster 2001, Mercier and others 1994). UV radiation can be divided into three parts: UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (200-280 nm). UV-A represents about 6% of the total solar radiation and is the least harmful part of UV radiation. UV-B can cause a variety of damaging effects in plants and it represents approximately 1.5% of the total spectrum. On the other hand, UV-C is very hazardous to organisms; however, the stratospheric ozone layer filters out most of this UV radiation (Hollosy 2002).

It is recognized that there are both direct and indirect effects of UV radiation on plants, which include changes in photosynthesis, cell division, change in DNA, and other processes related to growth and development. These effects are observed only after a few hours or days of exposure. Besides these damaging effects, plants also have "adaptive" responses, such as activation of different defensive mechanisms as protection against UV radiation. The most studied is the production of flavonoids, especially anthocyanins to screen out UV-B (Jenkins and others 1997).

Plants have to be able to deal with potentially harmful stress conditions that are almost constantly present in their environment. Since they cannot move, they have limited capability to avoid these stresses. Each cell must have mechanisms that allow any signals to be detected and acted upon to give rise to particular responses.

Plants exhibit physiological responses to protect them from damaging UV radiation. These responses include the synthesis of flavonoids, hydroxycinnamic acids and their related compounds. These responses are likely to involve specific UV photoreceptors and signal transduction processes, which lead to the regulation of gene

transcription (Jenkins and others 1997). Accumulation of these protective compounds is primarily in epidermal layers (Day and others 1993, Jenkins and others 1997). Flavonoids have λ_{max} at 270 and 345 nm, whereas hydroxycinnamic acids have λ_{max} at 320 nm, so both classes may protect against damaging UV radiation. Alternatively, UV radiation may interact with other atoms or molecules in the cell, particularly in water, which then produces free radicals (Kovacs and Keresztes 2002). These radicals can diffuse far enough to reach and harm different components of the cell. This effect is very significant in plants since the cytoplasm of plant cell contains about 80% water (Kovacs and Keresztes 2002). Plant cells may also respond to these reactive oxygen species by trigerring the phenylpropanoid metabolism (Carletti and others 2003).

Exposing the tissues to UV light was found to increase some phenolic compounds that have antioxidant property, such as anthocyanin in apples (Arakawa 1988, Dong and others 1995, Reay and Lancaster 2001), cherries (Arakawa 1993), resveratrol in grapes (Cantos and others 2001), and isocoumarins in carrots (Mercier and others 1994). All of these studies were characterizing the phenylpropanoid metabolism response, with the exception of the study of resveratrol in grapes which focused in the enhancement of the phenolic metabolism to improve the health benefit properties of the crop.

Most of the work found in the literature has been focused on the effects of only one particular stress. However, in nature, specific stress factors are often more subtle, occur over time and happen simultaneously with other stress or environmental changes (Stratman 2003). Therefore, there is a need to determine the effects and interactions among different stressor, applied simultaneously to tissues and the phenylpropanoid metabolism. In this chapter, we aim to study the application of a combination of wounding and different UV lights in carrot tissue and determine the response of the phenylpropanoid metabolism by measuring the total and individual phenolic compounds, the antioxidant capacity (AOX), and the PAL activity through time.

Materials and Methods

Plant materials and reagents

Carrots cultivar Legend, which were grown in California (Grimmby way Farms, Bakersfield, CA) were examined. This cultivar is commonly used for processing by the fresh produce industry. To decrease variability, all carrots used in the experiment were approximately the same size and were inspected for any visual damage because any injury prior to processing could induce PAL activity. The selected carrots were stored in 15^{o}C room until they were ready to be processed.

All chemicals and standards used: Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpyrrolidine (PVPP), sodium hydroxide (NaOH), boric acid, 2-mercaptoethanol, chlorogenic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and ferulic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol and acetonitrile were reagents of HPLC grade.

Wounding and UV radiation stresses

Carrots were processed after being washed with 100 ppm chlorinated water and dried at room temperature for 2-3 hours. For the first experiment, carrots were cut into slices, pies and shreds. Intensity of wounding, A/W of 4.2, 6.0, and 23.5 cm^2/g, respectively, were calculated based on our previous work (Surjadinata and Cisneros-Zevallos 2003) as mentioned in chapter II. Whole non-wounded carrots (A/W = 0 cm^2/g) were used as the control. These carrots were then exposed to 60 W of UV-C for 0, 0.5, 1, and 15 minutes at room temperature. The carrots were placed on weighing dishes on a single layer and were approximately 50 cm below the UV light. The carrots were inverted in the middle of the radiation process to achieve optimum exposure on all sides of the tissue.

For the second experiment, the carrots were only cut into pies and exposed to 120 W of UV-A or UV-B for 0, 1, and 6 h at room temperature. The cut carrots were also placed on weighing dishes on a single layer and were positioned 50 cm from the UV

light. The carrots were inverted in the middle of the radiation treatment for maximum UV exposure. The relative humidity of the UV chamber was kept above 90% by providing continuous humidified air flow onto the tissue. This was done to prevent moisture loss during radiation.

After treatment, the stressed carrots were placed in 4 L closed glass jars, stored in the dark at 15^{o}C, and ventilated every 8-12 h to avoid CO_2 accumulation. All treatments had 5 replicates of similar weight (around 150 g). Measurements were done after 4 d of storage.

Phenolics, AOX, and PAL activity

All quantifications were done according to Chapter II. Total phenolics were measured using Folin-Ciocalteau reagent, assayed spectrophotometrically at 725 nm. Individual phenolics were identified with reverse phase HPLC-DAD. Total phenolic content was expressed as mg chlorogenic acid equivalent/100g fresh weight tissue. Antioxidant capacity was measured using DPPH radical protocol, measured spectrophotometrically at 515 nm and expressed as μg Trolox equivalents/g fresh weight tissue. PAL activity was assayed with spectrophotometer at 290 nm and expressed as μmoles of *t*-cinnamic acid/h g fresh weight tissue.

Statistical analysis

Statistics analysis was done using the ANOVA procedure from the SAS Statistical Analysis System for Windows v8.1 (SAS Institute Inc, Cary, NC, USA). The treatment means were compared with Tukey's Studentized Range test at α=0.05.

Results and Discussion

Effect of UV-C and different wounding intensity (A/W) on total phenolic, PAL activity and AOX during storage

Total phenolics, AOX and PAL activity are all affected by wounding and exposure to UV-C light (Figure 12). There is significant increase ($P<0.05$) in phenolic

content compared to controls (non-wounded) and wounded tissues radiated with different UV-C exposure times.The interaction between wounding and UV-C stresses was synergistic since non-wounded tissue was basically not affected by the UV-C treatment. Compared to the non-radiated treatment, total phenolics in cut tissue increased 111, 143, and 15 %, for slices (A/W =4.2 cm^2/g), pies (A/W = 6.0 cm^2/g), and shreds (A/W = 23.5 cm^2/g), respectively, after exposed to UV-C for 15 minutes (Figure 12A). In general, for each wounding intensity, there was a dose response when UV-C was applied. The dose response decreased as follows: pies > slices > shreds > non-wounded. Apparently, at the highest wounding intensity, the tissue was less sensitive to UV-C. This would imply that a maximum phenolic synthesis may be reached by combination of stresses.

Plants have different response when it comes to different doses of UV radiation. They can either stimulate protection mechanisms or activate repair mechanisms to manage this stress (Frohnmeyer and Staiger 2003). The most common protective mechanism against these damaging effects is the biosynthesis of UV-absorbing compounds (Hahlbrock and Schell 1989), such as phenolics. These secondary metabolites are mainly accumulated in the vacuoles of epidermal cells to prevent further penetration of UV radiation into deeper cell layers (Frohnmeyer and Staiger 2003).

After 15 min of UV-C exposure, AOX increased 384, 506, and 37% for slices, pies, and shreds, respectively (Figure 12B). The trend followed by the increase in AOX was similar to that of phenolics for each wounding intensity. The oxidative burst cased by ROS has to be counteracted by antioxidants and protective pigments, such as flovonoids and carotenoids, to prevent cell damage (Carletti and others 2003). This could in part explain the increase in antioxidant capacity when the cut carrot tissues were radiated with UV-C.

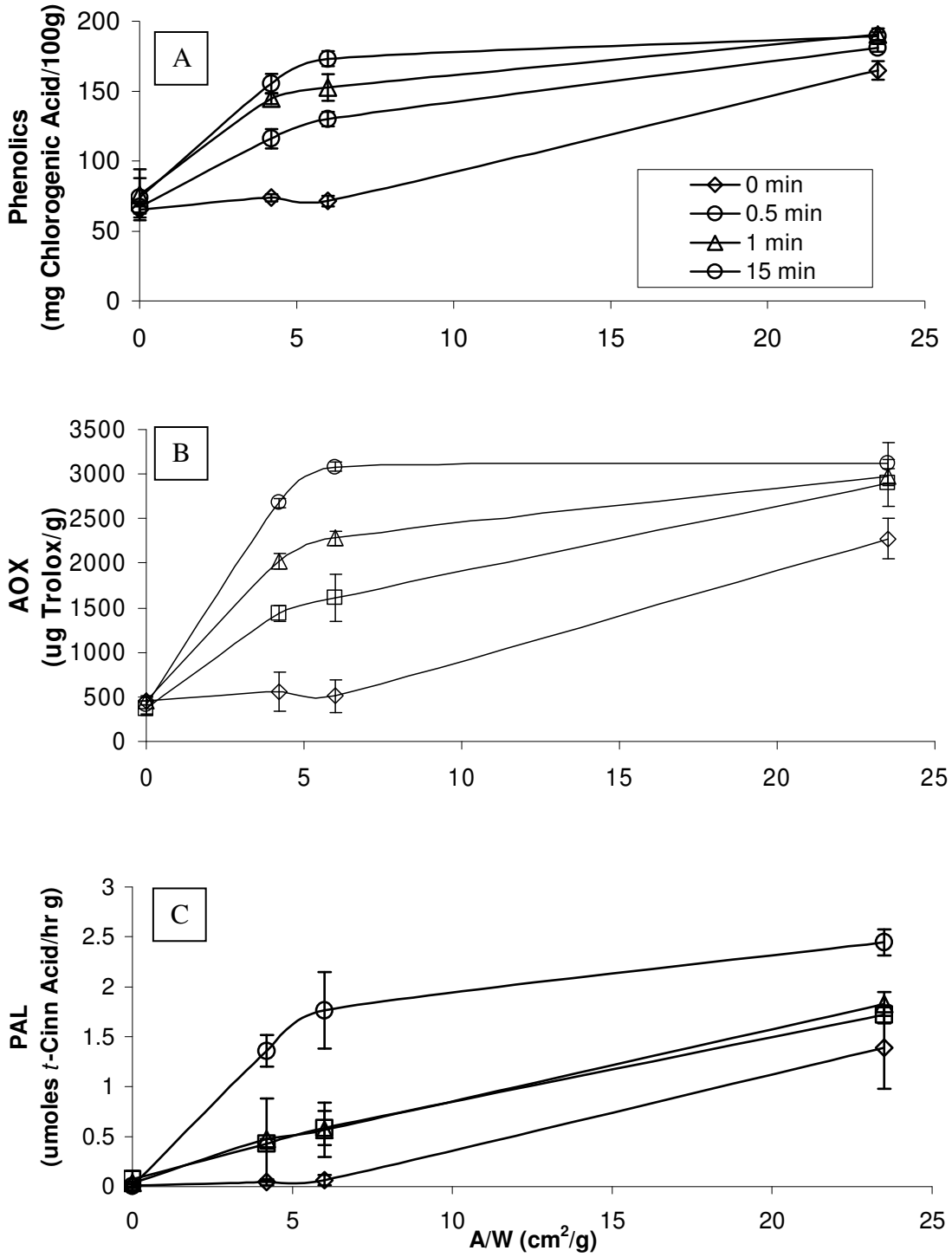

Figure 12 – Total phenolic content (A), antioxidant capacity (B), and PAL activity (C) of different cut carrots radiated with UV-C. Measurements were taken after 4 d storage at 15°C. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

ROS induction is known to be one of the early effects of exposure to UV lights. Vicente and others (2005) also found an increase in the antioxidant capacity of UV-C treated peppers stored at 10^{o}C for a period of 18 days.

PAL activity increased almost 30, 27, and 0.75 folds for slices, pies, and shreds respectively, after the cut tissues were radiated for 15 min of UV-C and stored at 15^{o}C for 4 days (Figure 12C). It is well established that light activates the expression of genes encoding PAL, CHS, and several other phenylpropanoid enzymes (Hahlbrock and Schell 1989, Takeda and others 1993, Jenkins and others 1997). Depending on the species, these enzymes are also regulated by different environmental and endogenous signals, such as abiotic stresses, pathogens, and plant growth regulators (Jenkins and others 1997).

Figure 12 also illustrates that non-wounded carrot tissues (A/W = 0 cm^2/g) radiated with UV-C did not show significant increase ($P>0.05$) in the total phenolics, AOX and PAL activity. It seems that for UV-C to have an effect on carrot tissue, there is a need of an initial wounding stress to take place. It has been shown that plants make decisions and prioritize for certain stress responses while down-regulating another response (McDowell and Dangl 2000, Saltveit 2000, Stratmann 2003).

Total phenolic in carrot tissue is highly related to AOX capacity. The linear relationship increased in this trend was observed for all wounding intensities (Figure 13). These shifts in the slope values are an indication that wounding and UV stresses synthesize different phenolic compounds with different AOX properties. For slices (Figure 13B) and pies (Figure 13C), the shifts in the slope values are more pronounce compared to shreds. These slopes are related to specific antioxidant capacity or the antioxidant capacity expressed on phenolic basis (Table 4). For non-wounded and shred carrots, the increase in UV-C radiation time did not mark a pronounce change in the specific antioxidant activity. However, for carrot slices and pies, the specific antioxidant activity increased significantly compared to non-UV-C radiated treatment. This means that UV-C stimulates the production of phenolics possessing higher bioactive antioxidants. This is proven to be true by the HLPC result in Table 5.

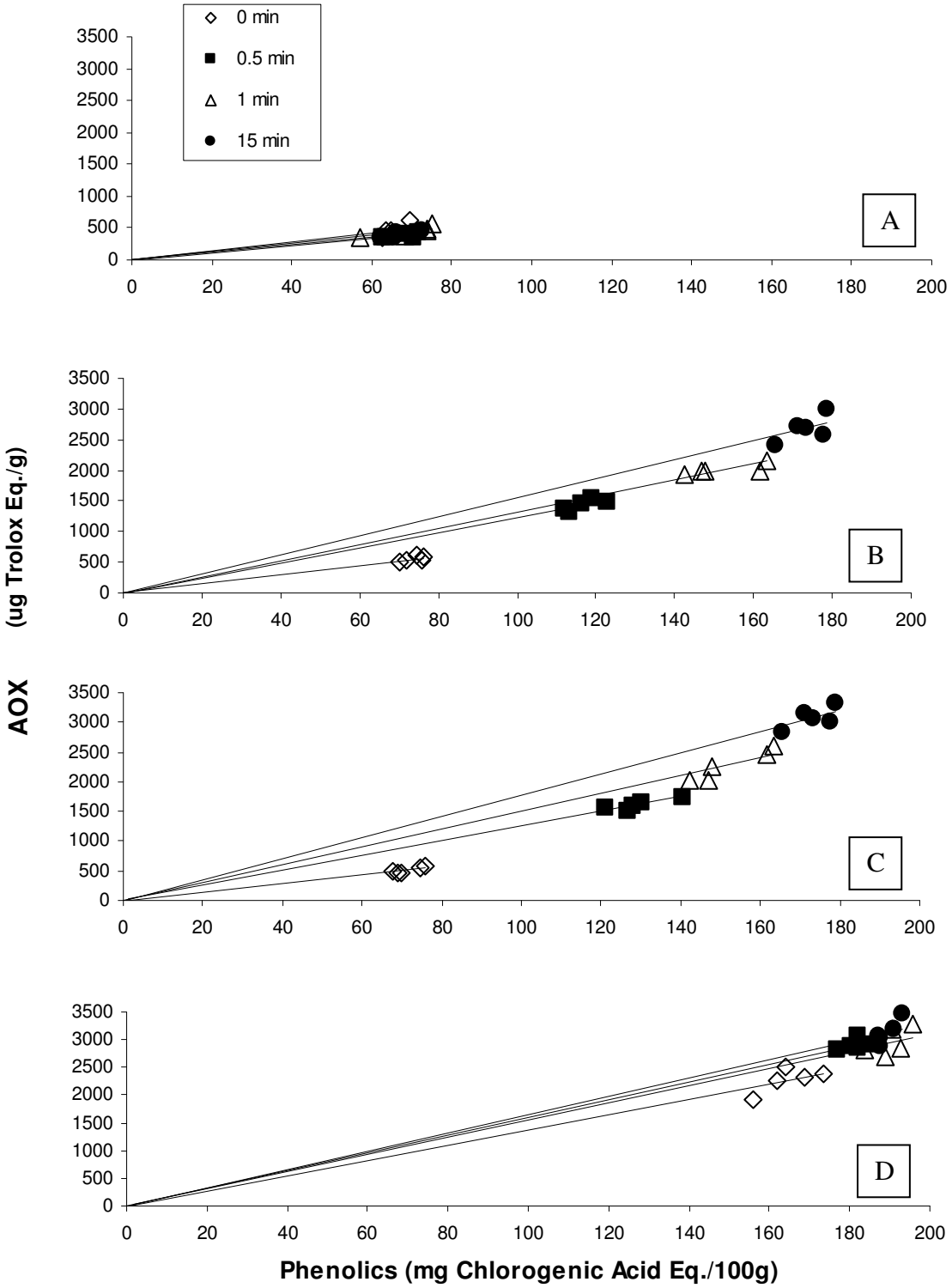

Figure 13 – Relationship between antioxidant capacity and total phenolic content on fresh weight basis of carrot tissue are linear for all UV-C treatments. (A) non-wounded, (B) slice, (C) pie, and (D) shred.

Table 4. Specific AOX (μg Trolox Eq./mg Chlorogenic Acid Eq.) ± SD (*n* = 5) of cut carrots radiated with UV-C.

Cut	A/W	UV-C (min)			
	(cm^2/g)	0	0.5	1	15
Whole	0.0	703 ± 121.15	555 ± 46.68	651 ± 77.15	601 ± 32.09
Slices	4.2	749 ± 50.63	1234 ± 49.43	1394 ± 51.60	1721 ± 96.27
Pies	6.0	712 ± 42.85	1239 ± 31.79	1495 ± 90.72	1776 ± 73.98
Shreds	23.5	1380 ± 106.24	1604 ± 43.59	1559 ± 119.12	1651 ± 96.64

From chapter II, we found that the HPLC phenolic profile of non-wounded carrots without any stress application showed only two major peaks: chlorogenic acid (5-CQA) and 3,5-dicaffeoylquinic acid (3,5-diCQA) (Figures 5-7). In this study, after treating the non-wounded carrots with 15 min of UV-C, the HPLC phenolic profile showed two additional peaks: ferulic acid (peak 4) and isocoumarin (peak 8) (Figure 14A). The HPLC profile of wounded tissue (slices and pies) without any UV radiation showed four major peaks: chlorogenic acid, ferulic acid, 3,5-dicaffeoylquinic acid, and isocoumarin (Chapter II, Figures 5-7). In this study, when the wounded tissue was treated with 15 min of UV-C, additional peaks appeared: *p*-hydroxybenzoic acid (peak 2), *p*-coumaric acid derivative (peak 3), and a hydroxybenzoic acid derivative (peak 7) (Figures 14B and C). All of these phenolic compounds were identified from the previous chapter, except for *p*-coumaric derivative (peak 3). This compound has very similar PDA spectra (Figure 16) compared to the *p*-coumaric acid standard (Figure 15). This result shows that combining stresses increases the total phenolic content and also synthesizes new compounds that were not detected in the original non-wounded or wounded tissue.

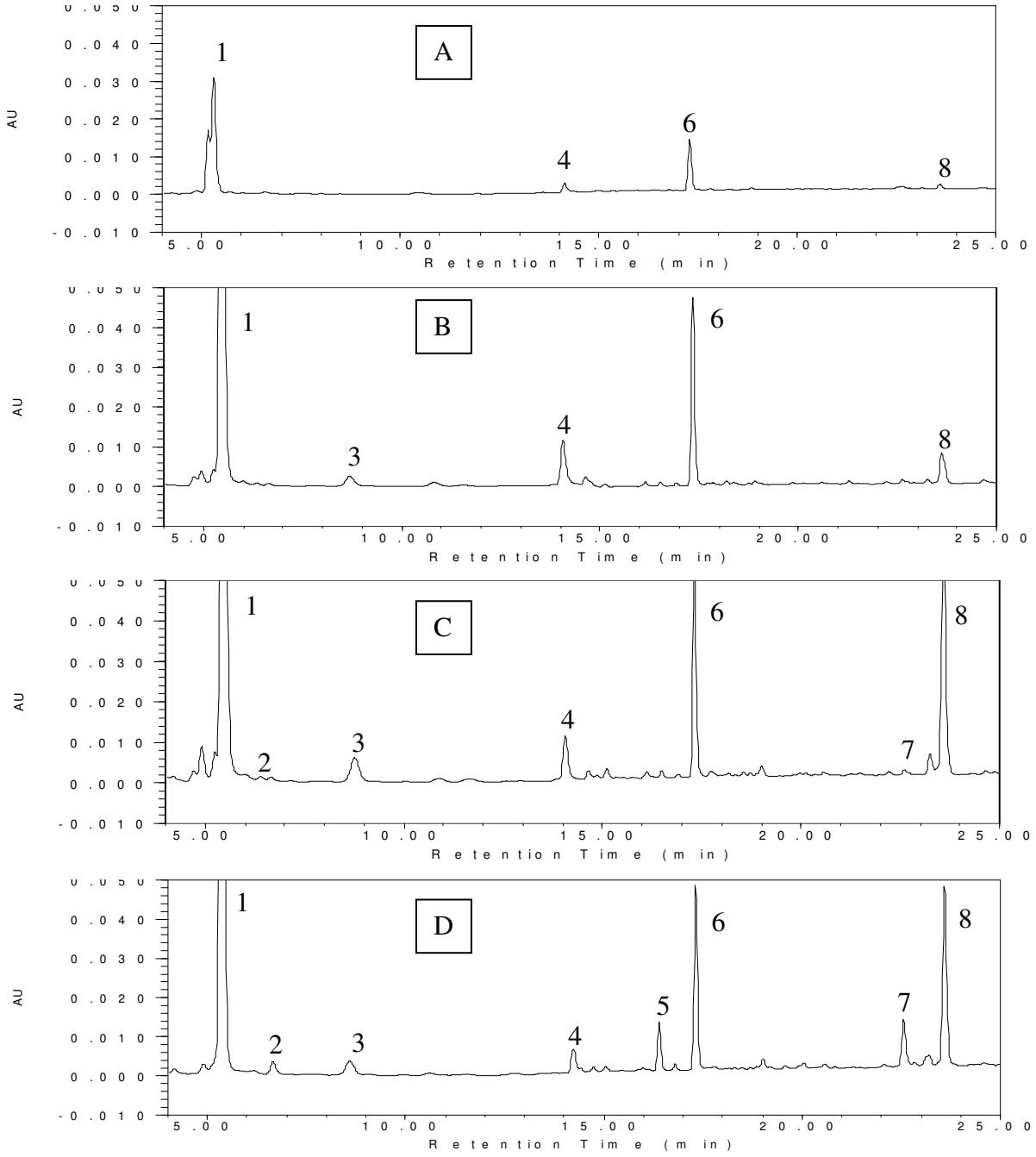

Figure 14 - HPLC phenolic profiles of non-wounded (A), slices (B), pies (C), and shreds (D) at 280 nm after 15 min of exposure to UV-C and 4 d storage at 15°C. Peaks: 1 = chlorogenic acid (5-CQA), 2 = *p*-hydroxybenzoic acid (*p*HBA), 3 = *p*-coumaric acid derivative, 4 = ferulic acid (FA), 5 = 3,4-dicaffeoylquinic acid (3,4-diCQA), 6 = 3,5-dicaffeoylquinic acid (3,5-diCQA), 7 = hydroxybenzoic acid derivative, 8 = isocoumarin. AU = absorbance unit.

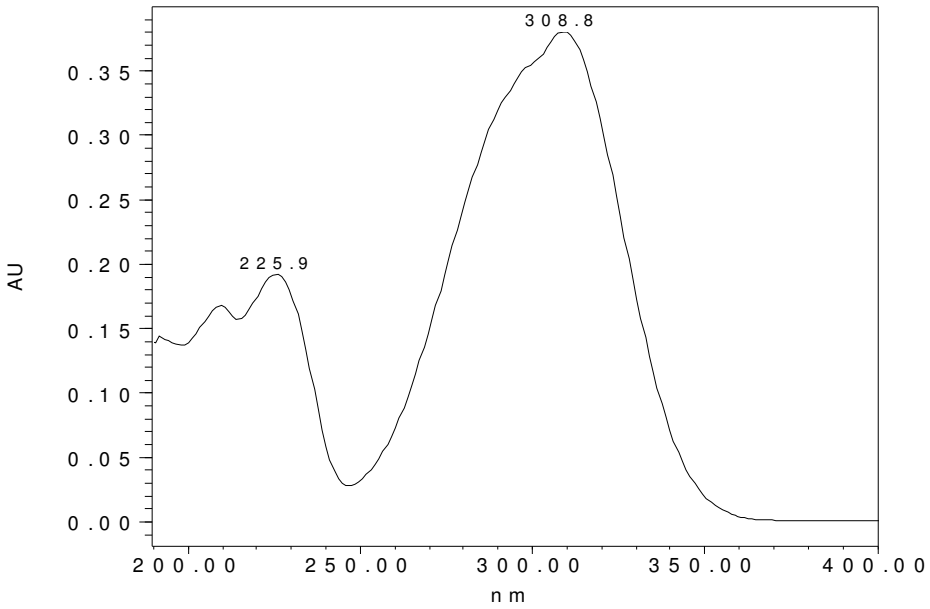

Figure 15 - Photodiode array (PDA) spectra of *p*-coumaric acid standard identified by HPLC at 280 nm. The retention time is 14.87 min. AU = absorbance unit.

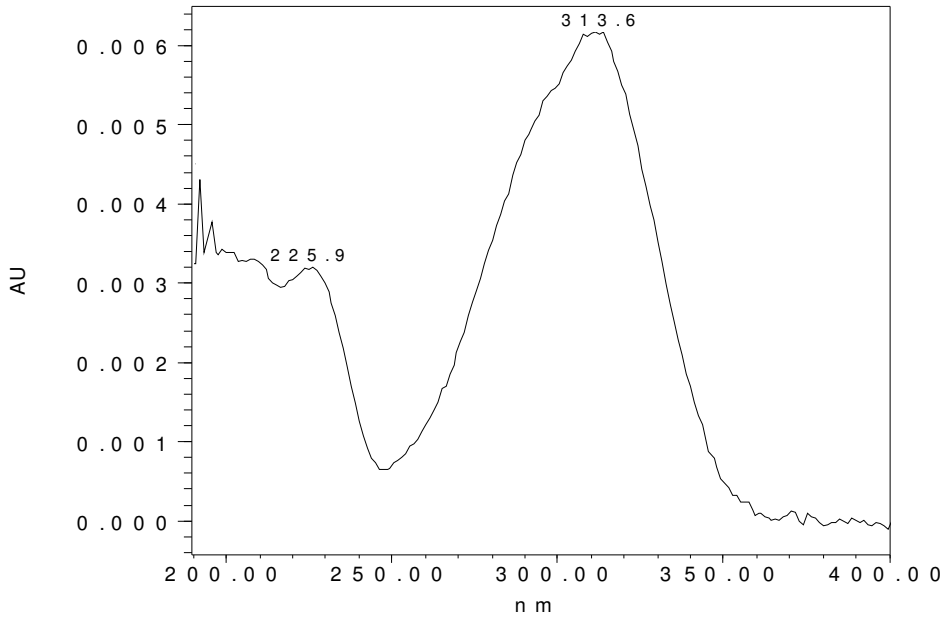

Figure 16 - Photodiode array (PDA) spectra of peak 3 = *p*-coumaric acid derivative identified by HPLC at 280 nm. The retention time is 8.62 min. AU = absorbance unit.

In all the UV-C treatments, the highest phenolic content was chlorogenic acid, followed by both diCQA isomers and ferulic acid (Table 5). According to Hudson and Mahgoub 1980, these hydroxycinnamic acid compounds have very potent antioxidant properties compared to hydroxybenzoic acids. Phenolics as antioxidants act as free radical scavenging by donating their hydrogen atom (Robbins 2003). If phenolics exert an antioxidant activity *in vivo* in plants, then the synthesis of hydroxycinnamic acids may play a role in scavenging ROS.

One of the concerns, that arise when carrots are stressed, is the increase in isocoumarin content since it could give a bitter flavor to the product (Lafuente and others 1996). According to Mercier and others (1994), the isocoumarin content of carrot slices increased when the tissue were radiated with UV-C. The authors related the increase in the phytoalexin isocoumarin with the inhibitory effect against *B. cinerea* fungal infection. In this study the intention is to keep the isocoumarin accumulation as low as possible so that the carrots would still have acceptable flavor quality. Calculated isocoumarin content reached a maximum of 2 mg/100g fresh weight under the stressed treatment (Table 5). This level is below the level for bitterness detection (20 mg/100g fresh weight tissue, Lafuente and others 1996). However, it is possible that the use of a more appropriate solvent, other than methanol, could have extracted more isocoumarin from the stressed tissue.

Wounded carrot exposed to different UV lights

Total phenolics, AOX capacity, and PAL activity of carrot pies were affected by exposure to UV-A and B lights. After 6 h of UV radiation, total phenolic content of carrot pies increased 1 and 3 folds, AOX capacity increased 2 and 7 folds, and PAL activity increased 34 and 90 folds, for UV-A and B, respectively (Figure 17). The dose response curve for UV-A, B, and C were different. Tissue response to the type of UV decreased as follows: UV-C > UV-B > UV-A.

Table 5 – Individual phenolic content (mg/100g FW) of non-wounded and cut carrots exposed to UV-C. Quantification was based on HPLC profile at 280 nm.

Peak	Compound	Whole radiated with UV-C (min)				Slices radiated with UV-C (min)				Pies radiated with UV-C (min)				Shreds radiated with UV-C (min)			
		0	**0.5**	**1**	**15**	**0**	**0.5**	**1**	**15**	**0**	**0.5**	**1**	**15**	**0**	**0.5**	**1**	**15**
1	5-CQA	10.00	8.45	9.61	11.17	12.00	26.99	40.80	52.12	9.21	27.01	36.19	75.52	44.70	60.71	65.42	58.78
2	*p*-HBA	nd*	nd	nd	0.56	nd	nd	nd	0.35	nd	nd	nd	1.16	nd	nd	nd	1.12
3	*p*-coumaric Derivative	nd	nd	nd	nd	nd	nd	nd	0.44	nd	nd	nd	0.86	nd	nd	nd	1.21
4	FA	1.77	1.71	1.83	1.60	1.95	2.25	2.82	3.24	1.79	2.29	2.38	2.38	2.49	2.64	2.52	2.31
5	3,4-diCQA	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	4.91	5.29	6.03	5.83
6	3,5-diCQA	6.64	7.26	7.41	6.36	6.61	7.74	10.77	12.02	6.37	8.13	10.19	12.45	13.23	13.57	14.00	12.27
7	HBA Derivative	nd	nd	nd	2.10	nd	nd	nd	1.44	nd	nd	nd	3.44	nd	nd	nd	3.58
8	Isocoumarin	0.04	0.05	0.04	0.04	0.06	0.55	0.23	0.30	0.06	0.16	0.92	1.84	0.59	1.07	1.45	1.31
	Total Phenolics**	**65.13**	**67.13**	**69.62**	**67.44**	**73.74**	**116.09**	**144.66**	**155.32**	**71.37**	**129.99**	**152.43**	**173.31**	**164.83**	**181.06**	**190.41**	**189.27**

* nd = not detected

** Total Phenolics were quantified based on mg Chlorogenic Acid equivalent/100g FW.

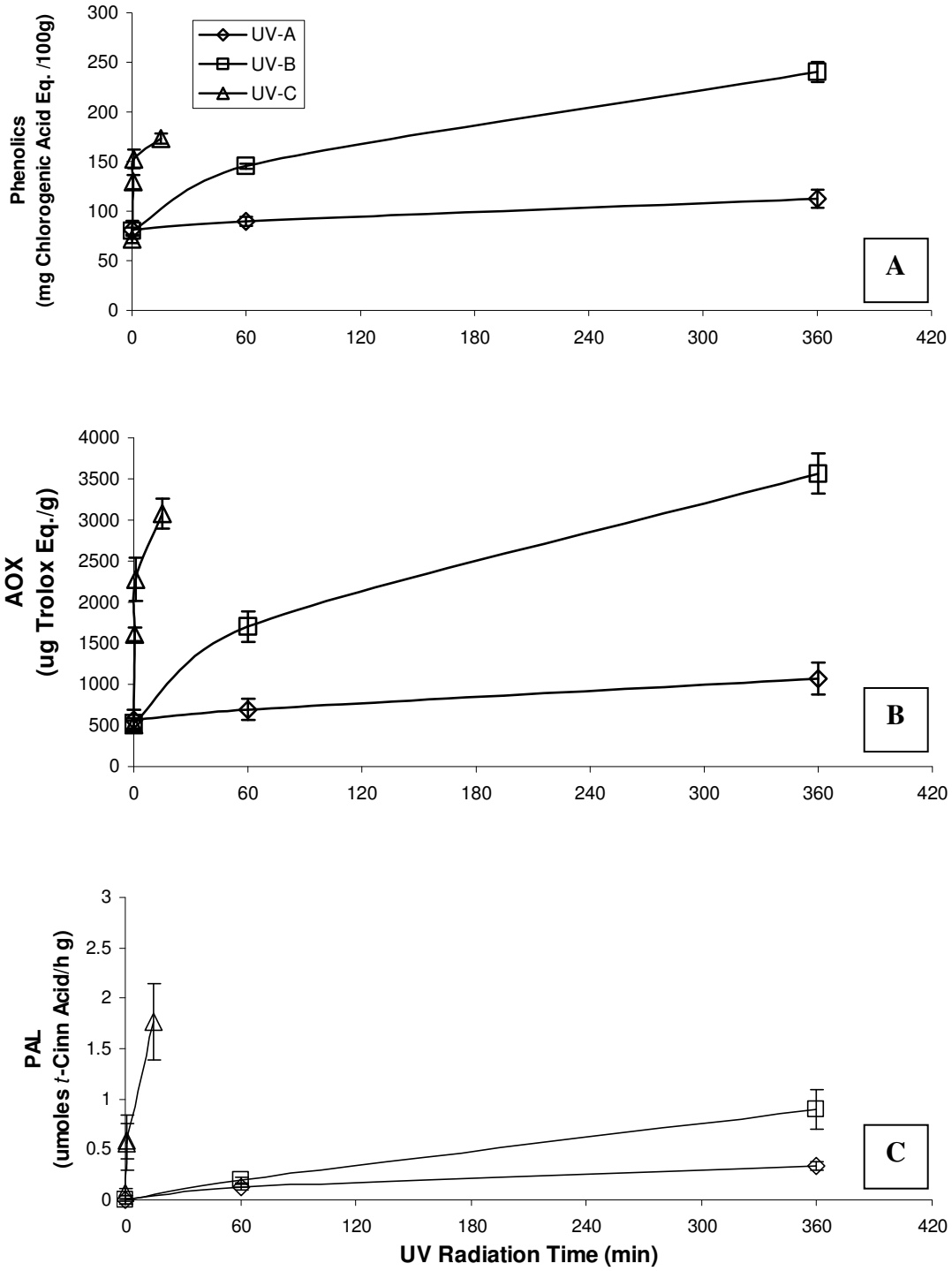

Figure 17 – Total phenolic content (A), antioxidant capacity (B), and PAL activity of carrot pies radiated with different UV lights. Measurements were taken after 4 d storage at 15°C. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

Wounded tissue exposed to different UV lights has different HPLC phenolic profiles. As shown in Tables 6, 7, Figures 18, and 19, the major compounds induced by wounding and UV-A or B were still chlorogenic acid and its hydroxycinnamic acids isomers (3,4- and 3,5- dicaffeoylquinic acids). Carrot pies exposed to 6 h of UV-A, showed all of the peaks shown by carrot pies exposed to UV-C, except for the presence of *p*-coumaric acid derivative (peak 3) and hydroxybenzoic acid derivative (peak 7) (Table 6 and Figure 18C). On the other hand, the carrot pies radiated with 6 h of UV-B showed all peaks, except *p*-hydroxybenzoic acid (peak 2) (Table 7 and Figure 19C). This result suggests the idea that different UV lights trigger different enzymes in the phenylpropanoid pathway. It has been well established that UV induces the expression of genes encoding phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) enzymes in trees (Julkunen-Tiitto and others 2005, Sullivan 2005). Takeda and others (1993) found that PAL mRNA accumulated in carrot cell suspension after radiation with UV-B. Dong and others (1995) found that activities of PAL and chalchone isomerase increased 10-20 folds after exposing Royal Gala apples to UV-B and white lights. Therefore, it would be important in the future, to study more phenylpropanoid enzymes, which can specifically trigger the synthesis of certain phenolic compounds when the tissue is subjected to UV radiation.

In general, the individual phenolic content induced by exposure to UV-B is higher compared to UV-A. A 6 h exposure of carrot tissue to UV-A or B only induced 0.12 and 1.56 mg isocoumarin/100g fresh weight tissue, respectively (Tables 6 and 7). These numbers are still below the maximum level for bitterness detection (Lafuente and others 1996). However, there is a need to test other solvents to ensure complete extraction of isocoumarin from the tissue matrix.

Table 6 – Individual phenolic content (mg/100g) of non-wounded and carrot pies exposed to UV-A. Quantification was based on HPLC profile at 280 nm.

Peak	Compound	Whole	Pie-cut radiated with UV-A		
			0 h	1 h	6 h
1	5-CQA	7.73	13.98	18.55	29.67
2	*p*-HBA	nd*	nd	nd	0.37
3	*p*-coumaric Derivative	nd	nd	nd	nd
4	FA	1.57	1.73	2.17	2.36
5	3,4-diCQA	nd	nd	nd	4.60
6	3,5-diCQA	4.93	5.35	5.74	6.85
7	HBA Derivative	nd	nd	nd	nd
8	Isocoumarin	0.05	0.11	0.10	0.12
	Total Phenolics**	**57.63**	**81.35**	**89.80**	**112.34**

Table 7 – Individual phenolic content (mg/100g) of non-wounded and carrot pies exposed to UV-B. Quantification was based on HPLC profile at 280 nm.

Peak	Compound	Whole	Pie-cut radiated with UV-B		
			0 h	1 h	6 h
1	5-CQA	7.82	13.15	38.84	70.63
2	*p*-HBA	nd*	nd	nd	nd
3	*p*-coumaric Derivative	nd	nd	0.33	0.38
4	FA	1.59	1.90	2.68	3.28
5	3,4-diCQA	nd	nd	nd	4.56
6	3,5-diCQA	4.95	6.42	7.95	13.33
7	HBA Derivative	nd	nd	nd	1.03
8	Isocoumarin	0.04	0.06	0.31	1.56
	Total Phenolics**	**67.47**	**79.84**	**145.21**	**240.44**

* nd = not detected

** Total Phenolics were quantified based on mg Chlorogenic Acid equivalent/100g FW.

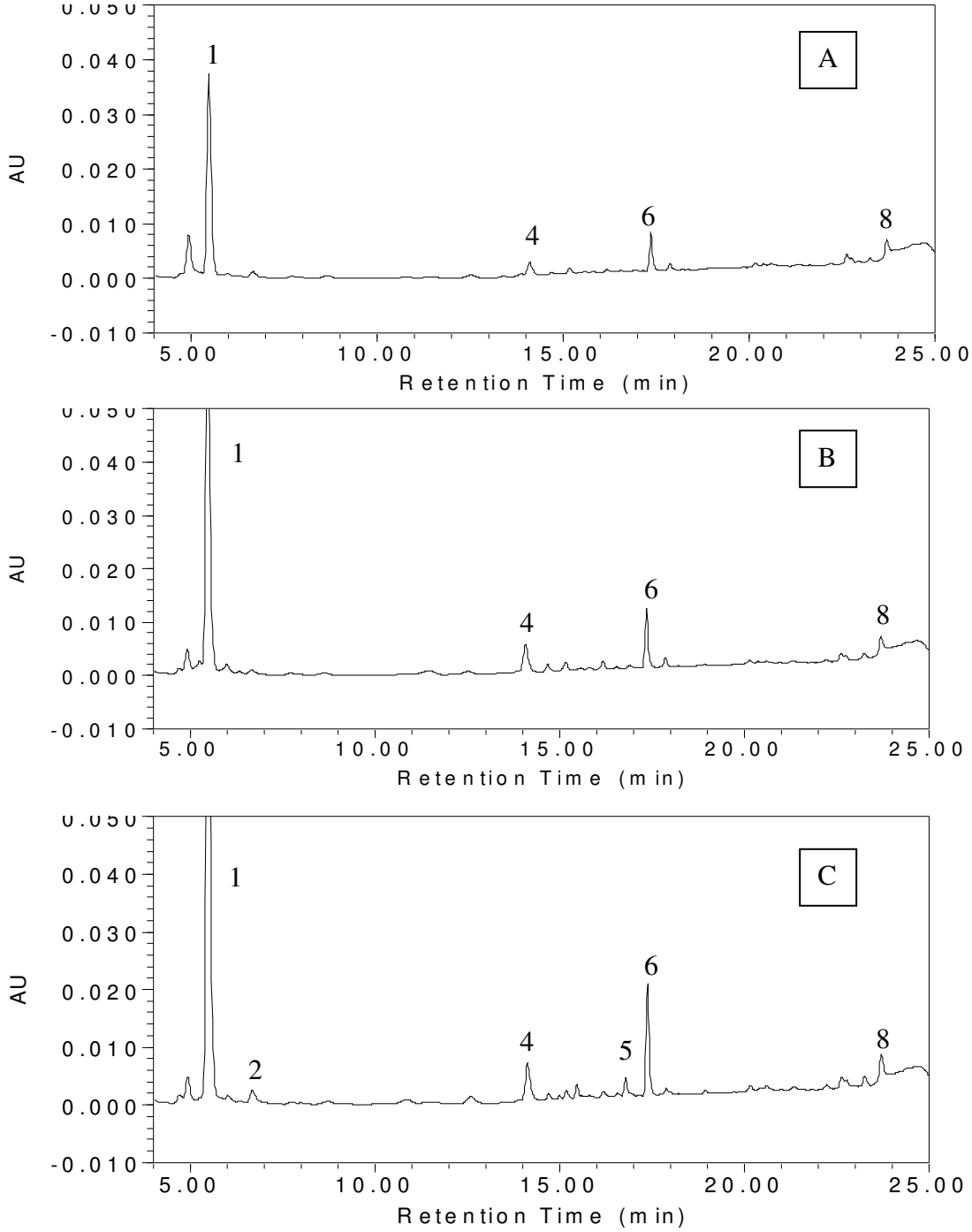

Figure 18 – HPLC phenolic profiles of carrot pies at 280 nm after exposure to 0 h (A), 1 h (B), and 6 h (C) of UV-A. Measurements were taken after 4 d storage at 15°C. Peaks: 1 = chlorogenic acid (5-CQA), 2 = *p*-hydroxybenzoic acid (*p*HBA), 3 = *p*-coumaric acid derivative, 4 = ferulic acid (FA), 5 = 3,4-dicaffeoylquinic acid (3,4-diCQA), 6 = 3,5-dicaffeoylquinic acid (3,5-diCQA), 7 = hydroxybenzoic acid derivative, 8 = isocoumarin. AU = absorbance unit.

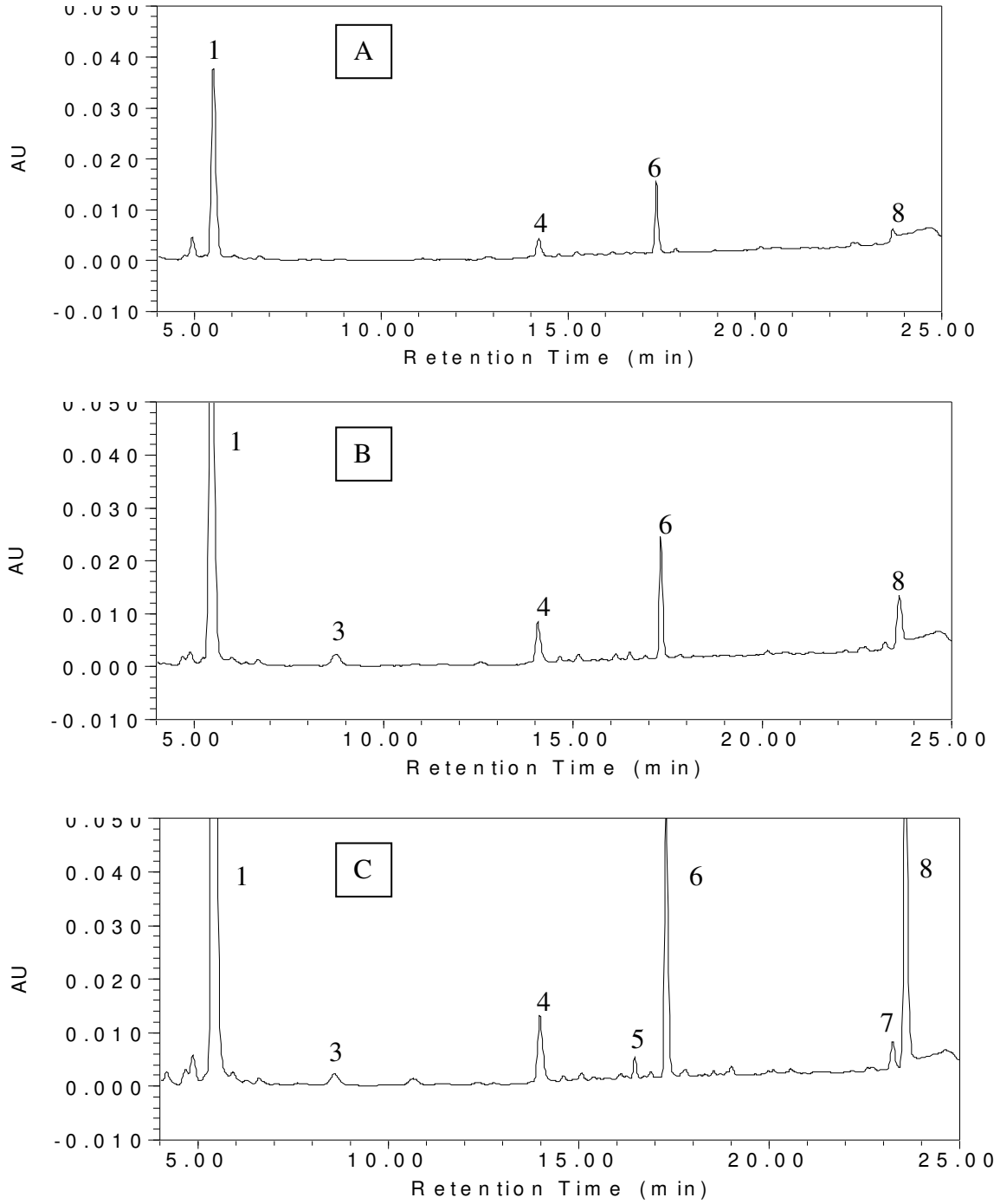

Figure 19 – HPLC phenolic profiles of carrot pies at 280 nm after exposure to 0 h (A), 1 h (B), and 6 h (C) of UV-B. Measurements were taken after 4 d storage at 15°C. Peaks: 1 = chlorogenic acid (5-CQA), 2 = *p*-hydroxybenzoic acid (*p*HBA), 3 = *p*-coumaric acid derivative, 4 = ferulic acid (FA), 5 = 3,4-dicaffeoylquinic acid (3,4-diCQA), 6 = 3,5-dicaffeoylquinic acid (3,5-diCQA), 7 = hydroxybenzoic acid derivative, 8 = isocoumarin. AU = absorbance unit.

The specific AOX increased as UV radiation time increased, which indicate that wounding stress combined with UV exposure induced phenolic compounds with more AOX capacity (Table 8). For tissue exposed to UV-A, there was no significant difference ($P>0.05$) in specific AOX at different U-A dose. However, for tissue exposed to UV-B, the specific AOX increased with UV-B dose (Table 8).

Table 8 also showed the relative proportions of the three major hydroxycinnamic acids present in the wounded carrot tissue exposed to different UV doses: chlorogenic acid (5-CQA), ferulic acid (FA), and one of the chlorogenic acid isomers (3,5-diCQA). The different UV doses give different relative proportions of 5-CQA : FA : 3,5-diCQA. FA and 3,5-diCQA proportions decreased with increased A/W. This means that the primary compound that determined the specific phenolic antioxidant is chlorogenic acid or 5-CQA.

Interestingly, when we combine the effect of all three UV lights together into one graph, we are able to see that certain phenolic compound was only triggered by certain UV light. Figure 20A shows that chlorogenic acid is synthesized by all UV lights, while Figure 20B shows that ferulic acid is induced by UV-A and B but not by UV-C. For 3,5-diCQA and isocoumarin, they are synthesized by UV-B and C, but not by UV-A (Figures 20C and D).

In general, our results show that exposing carrot tissue to different UV lights increase the phenolic contents. There are not many studies that have investigated the effect of UV-A in the phenolic contents of fruit and vegetable tissues. There are some studies that investigated the effect of UV-B on the synthesis accumulation of anthocyanin in light-colored sweet cherry (Arakawa 1993). Reay and Lancaster (2001) found similar result, where anthocyanins and quercetin glycosides were induced in Gala and Royal Gala apple fruit skin after radiation with both UV-B and visible lights.

Table 8 – Relative proportions of the three major hydroxycinnamic acids present (%) and the specific AOX ± SD (*n* = 5) of carrot pies tissue exposed to UV lights.

	Exposure Time (h)	5-CQA	FA	3,5-diCQA	Specific AOX (μg Trolox/mg Phenolic)*
UV-A	0	66.7	7.7	25.6	703 ± 93.87
	1	69.6	8.7	21.7	776 ± 119.74
	6	76.8	5.4	17.9	950 ± 118.15
UV-B	0	60.6	9.1	30.3	658 ± 50.63
	1	79.0	4.8	16.1	1173 ± 118.18
	6	80.3	4.5	15.2	1485 ± 49.44
UV-C	0	51.9	11.1	37.0	712 ± 42.85
	0.5 min	71.7	6.5	21.7	1239 ± 31.79
	1 min	75.0	4.2	20.8	1495 ± 90.72
	15 min	83.6	2.7	13.7	1776 ± 73.98

* Phenolic content was quantified based on 5-CQA spectrophotometric standard curve.

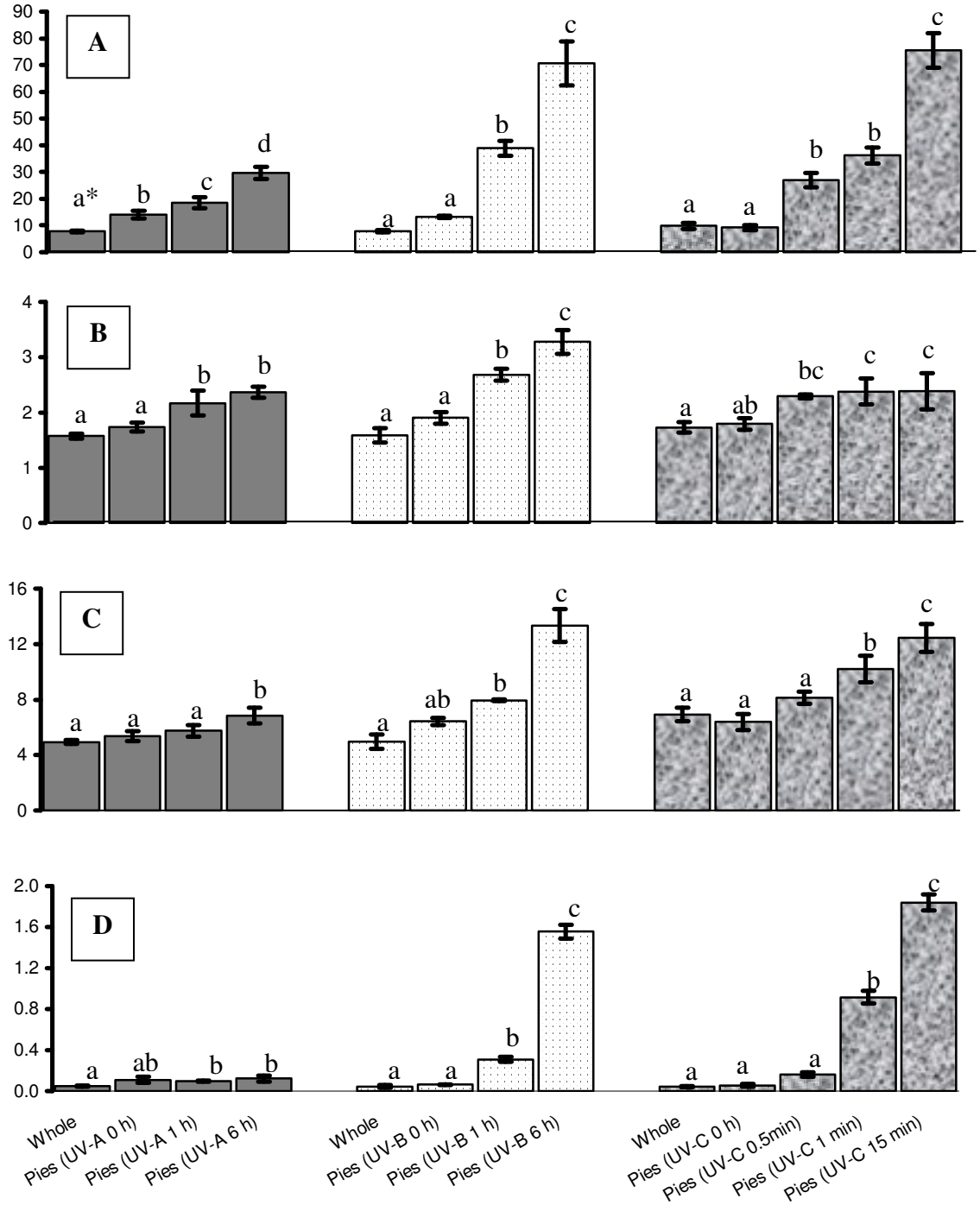

Figure 20 – Effect of different UV radiations on individual phenolic compounds of carrot tissue; chlorogenic acid (5-CQA) (A), ferulic acid (FA) (B), 3,5-dicaffeoylquinic acid (3,5-diCQA) (C), and isocoumarin (D). Y-axis is the phenolic content in mg/100g FW. Quantifications were based on HPLC profile at 280nm.

*Different letters show significant difference between treatment means (P<0.05).

Many studies have also been conducted to study the effect of UV radiations, mostly UV-B, on growing plants or trees. Many of these plants contain a wide variety of phenolics including cinnamic acids, chlorogenic acids, glycosylated flavonoids, and tannins (Julkunen-Tiitto and others 2005). They are present in all tissues, including leaves, stems, buds, flowers, and even roots. The existence of these secondary metabolites is fairly species-specific and plant-part specific. In addition, the relative composition may change dramatically over the development of the plant from seedlings to maturity (Julkunen-Tiitto and others 2005).

Most of these phenolics are UV-absorbing compounds. They play an important role in plant tolerance the UV radiation because they can reduce UV penetration into the plant tissue and also act as antioxidants to protect the plants from damage caused by ROS (Julkunen-Tiitto and others 2005). Lavola (1998) found that UV-B exposure to birch trees (*Betula pendula* Roth. and *Birch resinifera* Britt.) increased the amount of several flavonoids and phenolic acids when they were grown in a growth chamber or under greenhouse conditions. In general, the trees grown in the greenhouse have less phenolic compounds that responded to the UV radiation. This could be due to the higher PAR (photosynthetically active radiation) and lower fertilization (Julkunen-Tiitto and others 2005). Tegelberg and others (2003) showed variable induction of certain quercetins, dihydromyricetin, and phenolic acids in willow trees (*Salix myrsinifolia* Salisb.) after exposure of UV-B both in the field and indoor studies. Buffoni Hall and others (2002) found considerable increase (up to 154%) in the amount of phenolic compounds in the stem region of fruticose lichen (*Cladonia arbuscula* ssp. *mitis*) after exposure to UV-B radiation.

Potential signaling mechanism of UV radiation stress in carrot tissue

The mechanisms on how plants perceive UV radiation and initiate physiological responses are not fully understood. Most of the studies were done using UV-B radiation since the effect of UV-A is not significant in plants and most of UV-C radiation is

filtered out by the stratospheric ozone layer. Therefore, there is lack of information on the effects of UV-A and C on plants in the cellular and molecular levels.

Jenkins and others (1997) proposed several possible mechanisms for the specific detection of UV-B. The detection of signals in many cases is likely to involve specific cellular components called receptors (Jenkins and others 1997). Reception is coupled to the terminal response by signal transduction mechanisms. The signal transduction process often serves to amplify the initial signal and in some cases may store it for certain periods of time (Jenkins and others 1997).

The first proposed theory is that direct absorption of UV-B by DNA in the nucleus could result in the generation of some type of signals which stimulates the rate of transcription of several genes. The DNA molecule itself has been considered as a candidate for a UV-B receptor because many responses related to UV-B were maximally stimulated by wavelengths between 250 and 280 nm (Herrlich and others 1997). However, the action spectra of UV-B responses in plants revealed their maximal stimulation between 290 and 310 nm (Herrlich and others 1997). Additionally, there is a lack of correlation between the increase of DNA damage and UV-B elicited changes in transcript profile (Frohnmeyer and Staiger 2003). Therefore, these evidences contradict the theory that damaged DNA serves as UV-B receptor.

Other possible mechanism involves the detection of UV-B by photoreceptor molecules similar to other photoreception systems in higher plants. Plants have developed photo-sensory system that can monitor the change in their light environment. This system contains three known classes of photoreceptors: the phytochromes (PHY) for far-red and red lights, the crytochromes (CRY) for blue light, and the phototropins (PHOT) for UV-A light (Gyula and others 2003). The photoreceptor molecules for UV-B and C are still unidentified (Gyula and others 2003). In our situation, this proposed mechanism seems unlikely since carrot is a root. Naturally, carrot root grows underground, thus has no photoreceptor.

There are also other possible signaling molecules that are involved in the regulation of gene expression due to UV radiation. A-H-Mackerness and others (1999)

studied three different signaling pathways that control UV-B-induced stress on *Arabidopsis thaliana*: ROS, jasmonic acid, and ethylene. UV could be detected by the plant's ability to generate reactive oxygen species. In this case, the increase in transcription observed after UV exposure would be oxidative stress responses rather than photo-responses to UV wavelengths. The study by Green and Fluhr (1995) support this hypothesis. They found that UV-B response was greatly reduced when antioxidants were applied to tobacco leaves. The increase in AOX capacity of carrot tissue after wounding and UV radiation stresses in the present study could be linked to this possibility of ROS as the signaling molecule. UV-B exposure to *Arabidopsis* has been shown to induce activities of antioxidant enzymes system, such as guaiacol peroxidase, ascorbate peroxidase, and peroxidase (Rao and others 1996). On the other hand, applications of superoxide dismutase (SOD) and catalase (CAT), which are also components of the antioxidant enzyme system, have been proven to reduce the effect of UV-B on *Arabidopsis* (A-H-Mackerness and others 1999).

Besides ROS, another possible pathway activated by high doses of UV is linked to wound signal transductions, which involve jasmonic acid and ethylene. These molecules have been proposed to trigger the synthesis of phenolic antioxidants as a defense mechanism against these stresses. The role of ethylene in signal transduction pathways leading to gene expression in response to UV-B has been shown by A-H-Mackerness and others (1999) in *Arabidopsis* mutant and by Wang and others (2005) in leaves of maize seedlings.

However, there is also possibility that the above mechanisms are not solitary. It is feasible that UV-B regulates gene expression by combination of those mechanisms. According to A-H-Mackerness and others (1999), the increase in ROS levels after exposure to UV-B could lead to the increases in levels of jasmonic acid and ethylene. Overlap responses, such as these, have been shown to extend from the transcriptional level to the intercellular signaling pathways that regulate gene expression (A-H-Mackerness and others 1999, Stratmann 2003). As mention earlier, application of SOD and CAT enzymes has been shown to reduce the effect of UV-B on the gene transcripts

in Arabidopsis. This effect of SOD and CAT is likely through their activity at the surface of the cells, since they are unlikely to penetrate the plasma membrane (A-H-Mackerness and others 1999).

As mentioned in the previous chapter, the same signaling molecules (ROS, jasmonic acid and ethylene) involve in triggering wound-induced phenolic synthesis may also be the ones responsible when the tissue is exposed to UV lights. This proposed idea will be investigated and discussed in the next chapter.

Conclusions

Combining abiotic stresses, such as wounding and UV light radiation increased the phenolic content, antioxidant capacity, and PAL activity of carrot tissue. The increase was more significant and rapid when the tissue was exposed to UV-C for few minutes. Exposing the tissue to UV-B could also increase the phenolic content up to the same level when it was exposed to UV-C, but it takes more time to exert the response (hours, instead of minutes). As for radiation with UV-A, it also induced the synthesis of phenolic content, however, this increase was not as considerable as the other two UV lights.

Different wounding intensity (A/W) also affected the response of the tissue to UV radiation. The carrot pies have the highest synergistic effect of wounding and UV stresses, even though it's A/W is not as high as shredded carrots. There is a maximum amount of phenolics that can be synthesized and the contribution of one stress will depend on the contribution of the other.

The main phenolic compound induced by both wounding and UV radiation is chlorogenic acid. Combining these two stresses may selectively induce different phenolic profiles. Certain phenolic compound is only induced by particular type of UV light, while others can be elicited by all type of UV lights. These stresses could be used by the fresh-cut produce and food processing industries as tools to tailor the synthesis of desired phenolic compounds.

CHAPTER IV

THE ROLE OF JASMONIC ACID, REACTIVE OXYGEN SPECIES, AND ETHYLENE AS STRESS-INDUCED PHENOLIC SIGNALING MOLECULES IN CARROT TISSUE EXPOSED TO WOUNDING AND UV RADIATION

Synopsis

Plant secondary metabolites, such as phenolics, have been shown to increase when the tissue is subjected to abiotic stress. This abiotic stress could be caused by factors such as light, water, wounding, nutrients, temperature, and chemicals. Many studies have suggested a variety of signal molecules involved in the metabolism of these secondary metabolites. We focused our investigation on reactive oxygen species (ROS), jasmonic acid (JA), and ethylene (C_2H_4).

First, the production of those signaling molecules induced by wounding and UV-C stresses through storage and related to the synthesis of phenolics and its key enzyme (PAL) were measured. There was a series of outcomes that took place after the tissue was stressed. The first event that occurred soon after the stress is the production of ROS (O_2^- and hydrogen peroxide productions), then ethylene production, followed by the increase in lipoxygenase (LOX) enzyme activity which is related to an increase in the JA production. After a sequence of syntheses of these signaling molecules, then the cell started to elicit transcription factor to regulate gene expression of PAL enzyme, which eventually lead to the synthesis of phenolic compounds.

Second, we determined the contributions of ROS, JA, and C_2H_4 on stressed (wounding, UV-A, B, and C) carrot tissue. To achieve this objective, we applied different inhibitors that can specifically block the signaling pathways. DPI was used to inhibit NADPH oxidase, the enzyme that produces O_2^- and H_2O_2, phenidone was used to inhibit LOX, and 1-MCP was used to impede ethylene action. These inhibitors were applied either individually or in combinations. We found that ROS and JA show similar

importance when the tissue is subjected to wounding stress, while with UV radiation stress, ROS is likely to be the primary signaling molecule, followed by JA and ethylene.

Introduction

In plants, many compounds have been proposed to play a role in stress-induced signal transduction, such as phytohormones (ethylene, jasmonic acid, ABA), mitogen-activated protein kinases (MAPK), reactive oxygen species (ROS), systemin, oligosaccharides, as well as electrical pulses and hydraulic waves (Saltveit 2000, Leon and others 2001, Rakwal and Agrawal 2003). Most of these works only focused on the effects of one particular stress applied under extreme conditions. In nature, specific stress factors are often more subtle, occur over time and happen simultaneously with other stress or environmental changes (Stratman 2003). It is a challenging task to determine the interactions between multiple stressors. Therefore, from all of the signaling compounds mentioned above, it has not been possible to identify and define which one is the primary signal that triggers the stress-induced phenolic metabolism and whether or not they are independent on each other. Whatever the signal may be, a better knowledge of this signaling mechanism is becoming essential for further understanding of the effect of abiotic stresses on plant tissue.

Jasmonic acid (JA), through the octadecanoid pathway, is known to be one of the stress-induced signaling molecules (Leon and others 2001). It was reported that JA increased in different plants, only in minutes to several hours after the tissue was wounded (Rakwal and Agrawal 2003) and usually, both JA and ethylene are simultaneously required for the activation of wound signaling mechanism (Leon and others 2001). JA and its related compounds have been observed to be one of the primary signals in the production of plant secondary metabolites (Zhao and others 2003). Endogenous and exogenous JA and methyl jasmonates have shown to induce a wide variety of terpenoids, alkaloids, and phenylpropanoid compounds (Zhao and others 2003). Jasmonic acid is derived from octadecanoid pathway, originated from the polyunsaturated fatty acid (PUFA) α-linolenic acid (Westernack and Hause 2002). The

key enzyme of this pathway is lipoxygenase (LOX; EC 1.13.11.12). To investigate the role of JA as the possible signaling molecule, an inhibitor of this enzyme, such as phenidone can be used.

Ethylene biosynthesis is also shown to increase during stress condition, such as wounding, exposure to UV lights, drought, salinity, flooding, low and high temperatures (Lafuente and others 1996, Ecker 1995, Morgan and Drew 1997, Wang and others 2005). More data showed that stress-induced ethylene was involved in plant stress tolerance (Morgan and Drew 1997). Wound-induced ethylene is involved in the initial stress responses such as abscission, senescence, wound healing, and disease resistance, which then produces the defense-related compounds, such as phenolics (Masia 2003). When exogenous ethylene was applied to carrot root, the content of phenolics increased greatly, especially isocoumarin (Fan and others 2000, Heredia and Cisneros-Zevallos 2002). Ethylene has also shown to be induced in the early phase of UV-B radiation. Nara and Takeuchi (2002) has reported that ethylene might be involved in a signal transduction pathway that could activate the defense mechanism to UV-B radiation. However, there is only a few data available about how plants sense the UV-B radiation stress and how UV-B stimulates the ethylene synthesis (Wang and others 2005).

Application of ethylene inhibitor can be used to investigate the involvement of ethylene as stress-induced phenolic signaling molecule. 1-Methylcyclopropane (1-MCP) is an inhibitor of ethylene action. It binds to ethylene receptor such that ethylene cannot bind and elicit action (Blakenship and Dole 2003). Compared with ethylene, 1-MCP is active at much lower concentrations and its affinity for the receptor is approximately 10 times more than that of ethylene. 1-MCP will protect plant products from both endogenous and exogenous ethylene sources (Blakenship and Dole 2003). According to Sisler and Serek (1997), after a certain period, tissue treated with 1-MCP will resume its sensitivity to ethylene.

ROS was shown to be the key signaling molecule triggered by UV radiation stress (A-H-Mackerness and others 1999, Stratman 2003). Under mild stress conditions, ROS concentration can be kept minimal by the scavenging activity of enzymes and other

antioxidants (Hideg and others 2002). However, strong artificial stresses, as well as combination of several stress factors, are not capable to prevent or compensate the oxidative damage caused by ROS. Both superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are ROS that have been proposed to function as primary signals or messengers to elicit the production of antioxidants and ROS scavenging enzymes during acclimatization or exposure to mild stress in plant tissues (Purvis 2003). O_2^- and H_2O_2 have been reported to induce different plant secondary metabolites, such as furanocoumarin as phytoalexin in parsley cell culture, isoflavanoid glyceollin in soybean, *p*-coumaroyloctopamine in potato, and saponin in ginseng (Zhao and others 2005).

In plants, ROS can be generated from several sources, including NADPH oxidase, apoplastic peroxidase, and other oxidases in mitochondria, chloroplasts and peroxisomes (Zhao and others 2005). However, the ROS produced due to stress is mainly generated by NADPH oxidase (Zhao and others 2005). Therefore, by inhibiting this enzyme such as with diphenyleneiodonium chloride (DPI), it is possible to determine the participation of ROS. The inhibitory mechanism of DPI on NADPH oxidase has been proposed by O'Donnell and others (1993) and the potency of DPI was shown to be directly related to the rate of enzyme turnover (O'Donnell and others 1993).

A better perception of plant stress-induced signal transduction will aid in developing strategies to manipulate the production of phenolics, which could lead to the improvement of accumulation of desirable compounds or suppression of undesirable ones. In this part of the study, we intend to determine the role of ROS, JA, and ethylene as signal molecules in carrot tissue exposed to wounding and UV radiation, by using enzyme inhibitors or by inhibiting actions of the targeted signal molecules.

Materials and Methods

Plant materials and reagents

Carrots cultivar Choctaw grown in Bakersfield, California (Grimmway Farms, Bakersfield, CA) were used in this study. This cultivar is commonly used for processing by the fresh produce industry. All of the carrots were inspected and chosen from the best

qualities, such as size, no major damage, color, and not dehydrated, to reduce variabilities. The selected carrots were stored under refrigeration (0-5^{o}C) and conditioned in a 15^{o}C room overnight until they were ready to be processed.

Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), Trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH), polyvinylpyrrolidine (PVPP), sodium hydroxide (NaOH), boric acid, 2-mercaptoethanol, xylenol orange, sorbitol, epinephrine, sodium phosphate, sodium linoleic acid, Tris-HCl, L-phenylalanine, diphenyleneiodonium chloride (DPI) and sulfuric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate (NH_4SO_4), dimethyl sulfoxide (DMSO), phenidone, and hydrogen peroxide (H_2O_2) were purchased from Fisher Scientific International, Inc. (Pittsburgh, PA). Iron sulfate ($FeSO_4$) was purchased from Acros Organics (Pittsburgh, PA). Ethylene standard was purchased from Matheson Gas Product, Inc. (Newark, CA). 1-methylcyclopropene (1-MCP) tablets were kindly provided by AgroFresh, Inc (Spring House, PA). Methanol was reagent of HPLC grade.

Wounding and UV radiation stresses

For the first experiment, carrots were washed with 100 ppm chlorinated water and dried at room temperature for a few hours before processing. Then, they were cut into pies (A/W = 6.0 cm^2/g). These pies are carrots slices (5 mm thick and 2.5 cm in diameter) that were cut further into quarters. After cutting, the carrot pies were then placed on weighing dishes on a single layer and exposed to 60 W of UV-C for 15 minutes. The carrot tissues were positioned approximately 50 cm below the UV light and they were inverted once in the middle of the radiation process. This was done to assure maximum UV exposure. Carrot pies not exposed to UV-C were used as controls.

After processing, carrot pies samples were stored in 1 L closed glass jars in a 15^{o}C room in the dark. The jars were ventilated every 8-12 h to avoid anaerobic condition. Samplings were taken every 15 minutes for the first hour, then after 2, 4, 10, 24, 48, 72, and 96 h. Each measurement was replicated five times.

For the second and last experiment, after washing and drying, carrots were cut into shreds using a food processor (High Performance model, West Band Co., West Band, WI) without exposure to UV light or cut into pies and exposed to 120 W of UV-A or B for 6 h or 60 W of UV-C for 15 min. For UV radiation, the cut carrots were placed on a single layer on weighing dish and overturned to assure optimum exposure. During radiation process, the relative humidity of the UV chambers was kept above 90% to prevent dehydration. All of the radiation treatments were done at room temperature. Then, the carrots were treated with different inhibitors (describe below) and stored in 1 L closed glass jars in the dark at 15^{o}C. Measurements were taken at days 0 and 4.

Inhibitors

The second experiment was done to determine the right concentration of inhibitors/blockers that would be used for the third experiment. Specific inhibitor was applied to the stressed carrot tissue to inhibit the specific stress-induced signaling molecule. To inhibit LOX activity, which is the key enzyme in jasmonic acid production, 50 g of stressed carrots were dipped in a clear glass beaker containing 100 mL phenidone solution with different concentrations (0.1, 1 and 10 mM) for 2 min. The phenidone powder was first has to be dissolved in 2% methanol and then completed with nanopure water to achieve the desirable concentrations. To inhibit NADPH oxidase, which could produce superoxide radicals and hydrogen peroxide, 50 g of stressed carrots were dipped into a clear glass beaker containing 100 mL of DPI (diphenyleneiodonium chloride) solution for 2 min. The DPI concentrations used were 3.17, 31.7, and 317 μM. Every gram of DPI powder was dissolved in 0.1 mL DMSO (dimethyl sulfoxide) before completion with nanopure water to reach the desired concentrations. To block ethylene action, whole non-stressed carrots were exposed to 1-MCP (1-methylcyclopropene) at 15^{o}C for 12 h. Different concentrations of 1-MCP used were 0.1, 0.5, and 2.0 μL/L. Application of 1-MCP (SmartFresh^{TM}) was done in a 31 gallon closed container equipped with a fan, following instructions for use of the SmartFresh^{TM} tablets provided by AgroFresh, Inc. (Spring House, PA). Two controls were used: stressed carrots

exposed to air or dipped into nanopure water for 2 min. Reading on initial day was also taken for carrots that were neither stressed nor subjected to any inhibitors.

For the third experiment, stressed carrots were subjected to the highest concentration of inhibitors mentioned above, either individual or combination of two or all three of the inhibitors (Table 9). The applications of the inhibitors are shown in Figure 21.

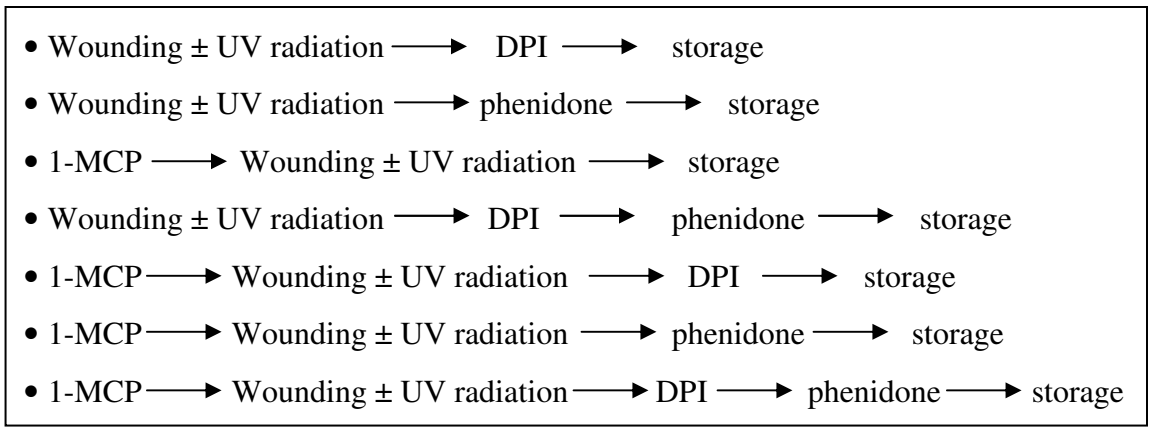

Figure 21 – Procedures of signal molecule inhibitors application, either individual or in combinations.

Phenolics, AOX, and PAL activity

All quantifications were done according to Chapter II. Total phenolics were measured using Folin-Ciocalteau reagent, assayed spectrophotometrically at 725 nm. Individual phenolics were identified with reverse phase HPLC-DAD. Total phenolics were expressed as mg chlorogenic acid equivalent/100g fresh weight tissue. Antioxidant capacity was measured using DPPH radical protocol, measured spectrophotometrically at 515 nm and expressed as μg Trolox equivalents/g fresh weight tissue. PAL activity was assayed with spectrophotometer at 290 nm and expressed as μmoles of *t*-cinnamic acid/h g fresh weight tissue.

Table 9 – Combination inhibitors experimental design.

Shreds	DPI 317 µM	PND 10 mM	1-MCP 2.0 µL/L	DPI 317 µM + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM	1-MCP 2.0 µL/L + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM + PND 10 mM
	Air Control	Water Control 2 min	Water Control 4 min				
Pies + UV-A	DPI 317 µM	PND 10 mM	1-MCP 2.0 µL/L	DPI 317 µM + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM	1-MCP 2.0 µL/L + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM + PND 10 mM
	Air Control	Water Control 2 min					
Pies + UV-B	DPI 317 µM	PND 10 mM	1-MCP 2.0 µL/L	DPI 317 µM + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM	1-MCP 2.0 µL/L + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM + PND 10 mM
	Air Control	Water Control 2 min					
Pies + UV-C	DPI 317 µM	PND 10 mM	1-MCP 2.0 µL/L	DPI 317 µM + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM	1-MCP 2.0 µL/L + PND 10 mM	1-MCP 2.0 µL/L + DPI 317 µM + PND 10 mM
	Air Control	Water Control 2 min					
Pies	Air Control	Water Control 2 min					
Day 0							

Ethylene concentration

Stressed carrots (75 g) were placed inside 1 L clear glass jars. These carrots were allowed to accumulate ethylene for 1 h in the closed system and gas samples were withdrawn using 1 mL syringes. The amount of ethylene in each treatment was measured by injecting the gas sample manually into a gas chromatography system (Photovac 10S Plus, Waltham, MA, USA).

This system is equipped with a Total VOC (Volatile Organic Compound) detector and an encapsulated capillary column. Ultra zero air was used as the carrier gas. Concentration of ethylene is reported as pmoles of ethylene per g of fresh weight tissue per h based on a developed standard curve.

Lipoxygenase (LOX) activity

Extraction and LOX assay was determined by method of Wang and others (2005). Five grams of fresh tissue was combined with 0.5 g of PVPP and 25 mL of cold extraction buffer, which contained of 100 mM Tris-HCl (pH 8.0), then homogenized at low speed until reaching uniform consistency. The Tris-HCl buffer was prepared with nanopure water. The extract was centrifuged at 30,000 *g* for 50 min at 4^{o}C. The clear supernatant was used directly for the assay. Throughout the analysis, samples were kept in ice and dark condition to prevent enzyme activation and protein denaturation.

LOX activity was quantified by measuring the formation of conjugated dienes at 234 nm at 30^{o}C. The reaction mixture solution contained 2.85 mL of 100 mM sodium phosphate buffer (pH 6.0), 50 µL of 10 mM sodium linoleic solution, and 0.1 mL of sample supernatant. At the same time a blank of 2.95 mL of 100 mM sodium phosphate buffer (pH 6.0) and 50 µL of 10 mM sodium linoleic solution was prepared. Both sodium phosphate buffer and sodium linoleic solution were prepared with nanopure water. The increase in absorbance at 234 nm was measured spectrophotometrically (Hewlett-Packard 8425A, Waldbronn, Germany) after incubating the sample mixtures in 30^{o}C water bath for 30 min. LOX activity is expressed as µmol/per gram of fresh weight tissue per minute. This calculation was done using a developed standard curve.

Superoxide (O_2^-) radical production

Extracellular production of O_2^- radicals was estimated by using the protocol of Beckett and others (2004) with slight modifications. It has been shown that O_2^- causes the oxidation of epinephrine to adenochrome which exhibits an absorption maximum at 480 nm and has an extinction coefficient of 4020/M cm (Misra and Fridovich 1972). Ten grams of carrot pies sample was mixed with 40 mL of 1 mM epinephrine (pH 7.0) in flask. The epinephrine powder was first dissolved in 0.6 M HCl (hydrochloric acid) and completed with nanopure water to achieve the final concentration of 1 mM. The flasks containing the tissue sample and the epinephrine solution were shaken at 120 RPM for 15 min in the dark at room temperature. The absorbance of the incubation solution is measured with spectrophotometer (Shimadzu UV-12015, Australia) at 480 nm. Production of O_2^- radicals is expressed as μmol O_2^- per gram of fresh weight tissue per hour.

Hydrogen peroxide (H_2O_2) production

H_2O_2 was assayed using the method of Beckett and others (2004) with slight modifications. This assay measures hydrogen peroxide concentration based on the oxidation of ferrous iron in acid solution and the formation of a colored complex between ferric iron generated and xylenol orange (Gay and Gebicki 2000).

Two reagent mixtures were prepared. Reagent A consisted of 25 mM of $FeSO_4$, 25 mM $(NH_4)SO_4$ and 25 mM of H_2SO_4. Reagent B consisted of 125 mM xylenol orange and 100 mM sorbitol. Both reagents A and B were prepared with nanopure water to attain the correct concentrations. The working reagent was comprised a mixture of reagents A and B (1:100 v/v). For each replicate, 10 g of carrot pies tissue was incubated in a flask containing 40 mL of nanopure water and shaken at 120 RPM for 15 min in dark condition at room temperature. 300 μL of the incubation solution was mixed with 2700 μL of the working reagent and let to react for 15 min in dark condition at room temperature. H_2O_2 production was quantified spectrophotometrically at 560 nm

(Hewlett-Packard 8425A, Waldbronn, Germany) and expressed as μmol H_2O_2 per gram of fresh weight tissue per hour.

Statistical analysis

Statistics analysis was done using the ANOVA procedure from the SAS Statistical Analysis System for Windows v8.1 (SAS Institute Inc, Cary, NC, USA). The treatment means were compared with Tukey's Studentized Range test at α=0.05.

Results and Discussion

Baseline study of phenolics and PAL activity under wounding stress

In this part of the study, the possible stress-induced signaling molecules related to the synthesis of phenolic compounds were quantified, including ethylene, jasmonic acid and ROS. Lipoxygenase enzyme activity was measured as an alternative for jasmonic acid quantification. Lipoxygenase is the first key enzyme in the octadecanoid pathway, which synthesizes jasmonic acid. Superoxide radical and hydrogen peroxide productions were measured as they are related to ROS. Additionally, ethylene production, total phenolics, and PAL activity were also measured. Some of these signaling molecules could be maximally produced only after a few minutes. Therefore, it is important to monitor them after a short period of time and also throughout storage. The result from previous chapter showed that carrot pies have the highest synergistic effect when wounding was combined with UV-C radiation stress. Therefore, to reduce the amount of samples, only carrot pies ± UV-C was analyzed.

Figure 22 shows that total phenolic content in both carrot pies and UV-C treated tissue increased considerably through time. However, this increase did not start until 10 h after the stress and it became more significant ($P<0.05$) with storage time. This result is similar to that of chapter II and to the findings by Alasalvar and others (2005). Their study showed that accumulation of phenolic compounds increased with storage time at 2-5^{o}C in shredded carrots.

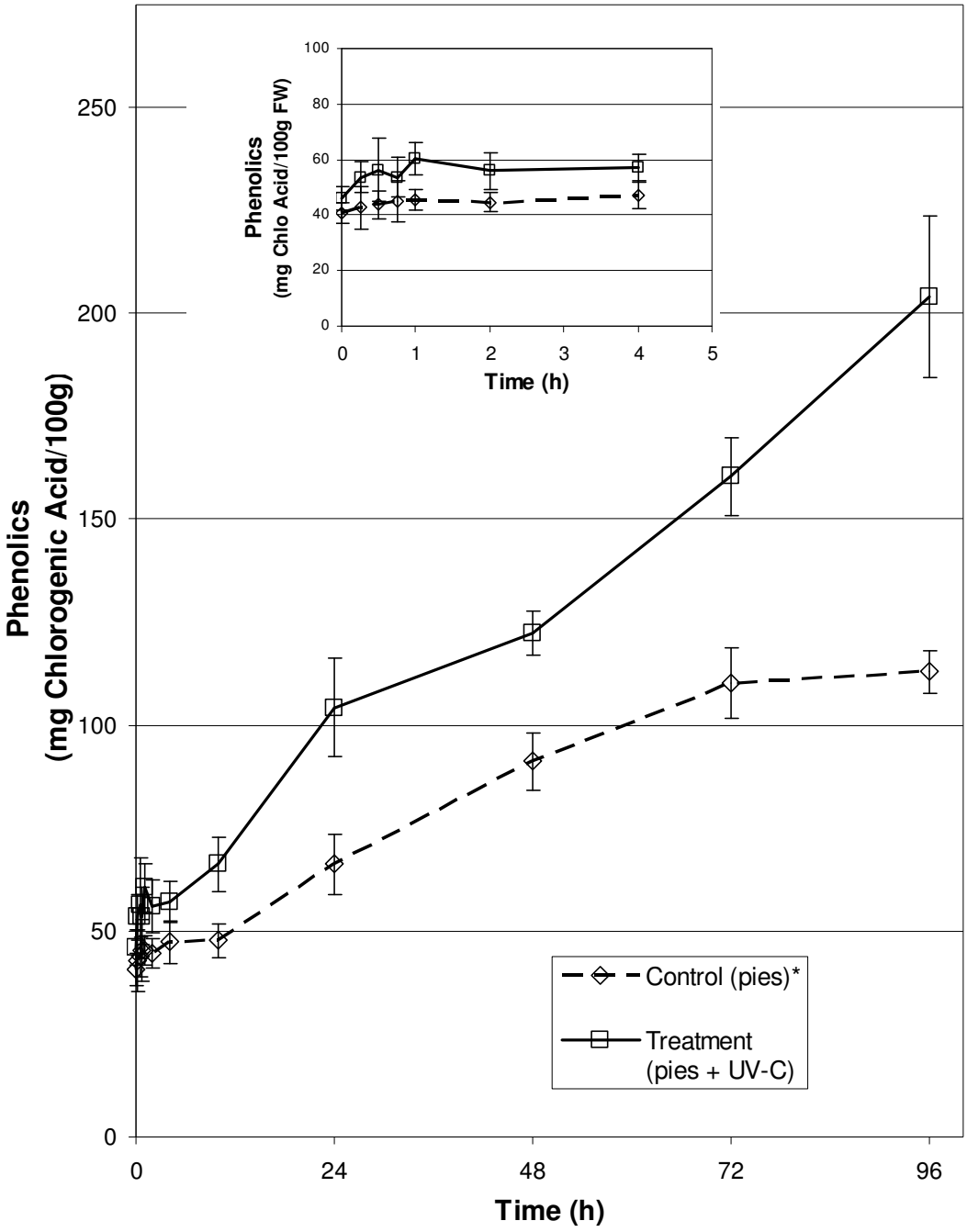

Figure 22 – Total phenolic content of carrot pies ± UV-C through time. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* Control and treatment are significantly different ($P<0.05$).

Combining wounding with UV-C radiation enhanced the synthesis of phenolic compounds. After 4 d storage, total phenolics for the carrot pies samples increased 277% compared to day 0 and the total phenolics for the UV-C treated samples increased 444%. Vincete and others (2005) found that total phenolics of pepper fruits increased during storage for both the control (no UV-C) and the UV-C treated samples. Cantos and others (2001) reported an increase in resveratrol content of grape skins after exposure to UV-C. Another study by Mercier and other (1994), determined that carrots radiated with UV-C induced the accumulation of the phytoalexin isocoumarin.

Wounding and the UV-C treatment also induced PAL activity during storage (Figure 23). For carrot pies, PAL reached a maximum activity after 10 h and then decreased, reaching steady state after 1 d. For UV-C treated samples, PAL reached its maximum activity after 24 h and decreased afterwards. The decline in PAL activity may be due to the presence of both a PAL-synthezising and a PAL-inactivating system (Zucker 1968, Chalutz 1973, Creasy and others 1986). PAL activity increased 1014% and 1856%, for carrot pies and UV-C treated samples, respectively. Even though PAL activity decreased after reaching a peak, total phenolics remained increasing due to the fact that PAL activity are still higher than the level of day 0.

Production of superoxide radical and hydrogen peroxide

Superoxide radical (O_2^-) production was monitored through time for both the carrot pies and UV-C treated samples (Figure 24). During the first 4 h after the stress, O_2^- production for the UV-C treated carrots was higher ($P<0.05$) than the carrot pies. However, after 4 h, there was no significant difference ($P>0.05$) between treatments. Both carrot pies and UV-C treated samples showed a maximum peak after 15 minutes and then a rapid decline afterwards until reaching steady state. Beckett and others (2004), studying a desiccated-stress plant tissue, found that O_2^- and H_2O_2 productions initially increased, reaching maximum peak after 15 minutes, similar to the present work.

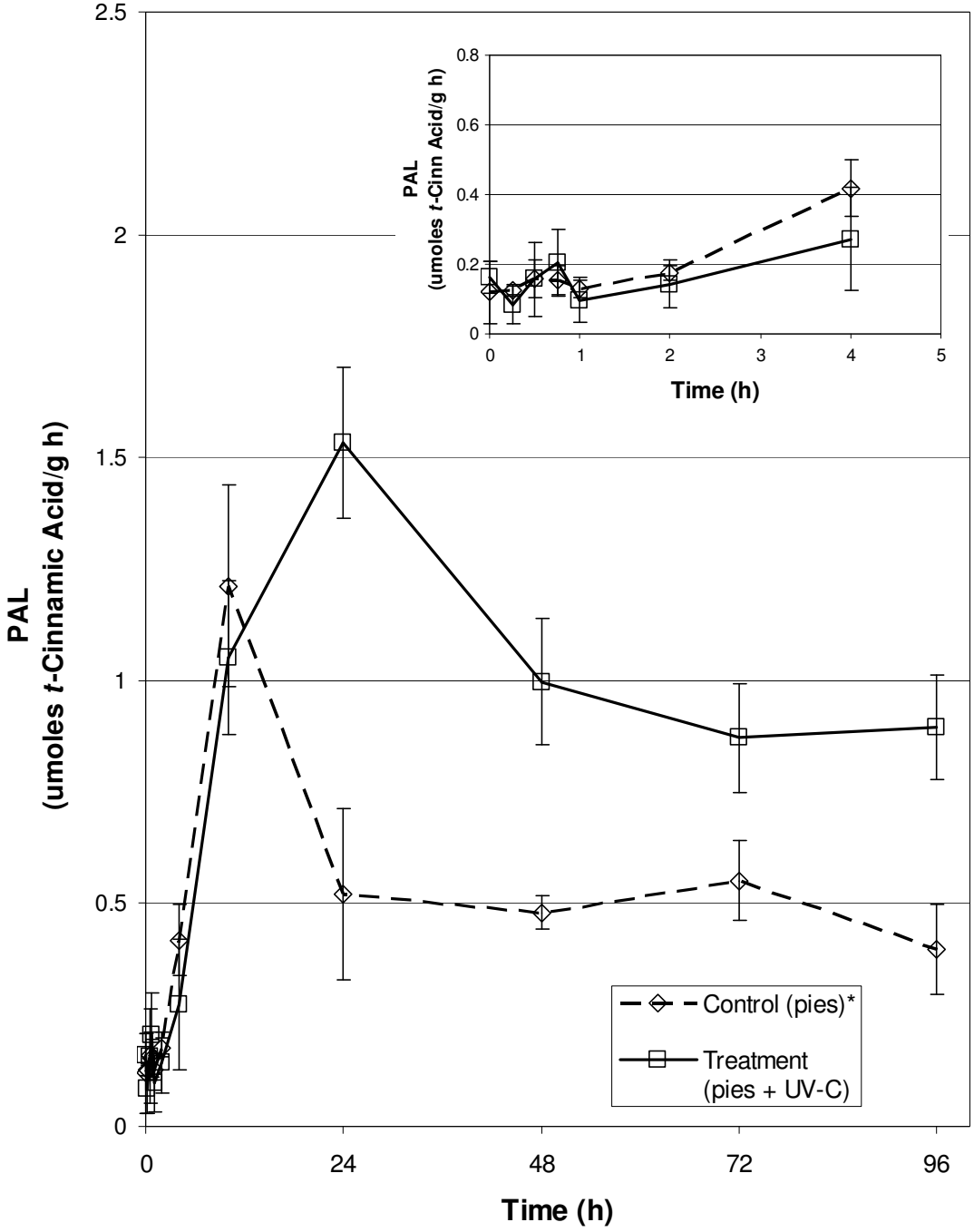

Figure 23 – PAL activity of carrot pies ± UV-C through time. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* Overall means for the control and treatment are significantly different (P<0.05).

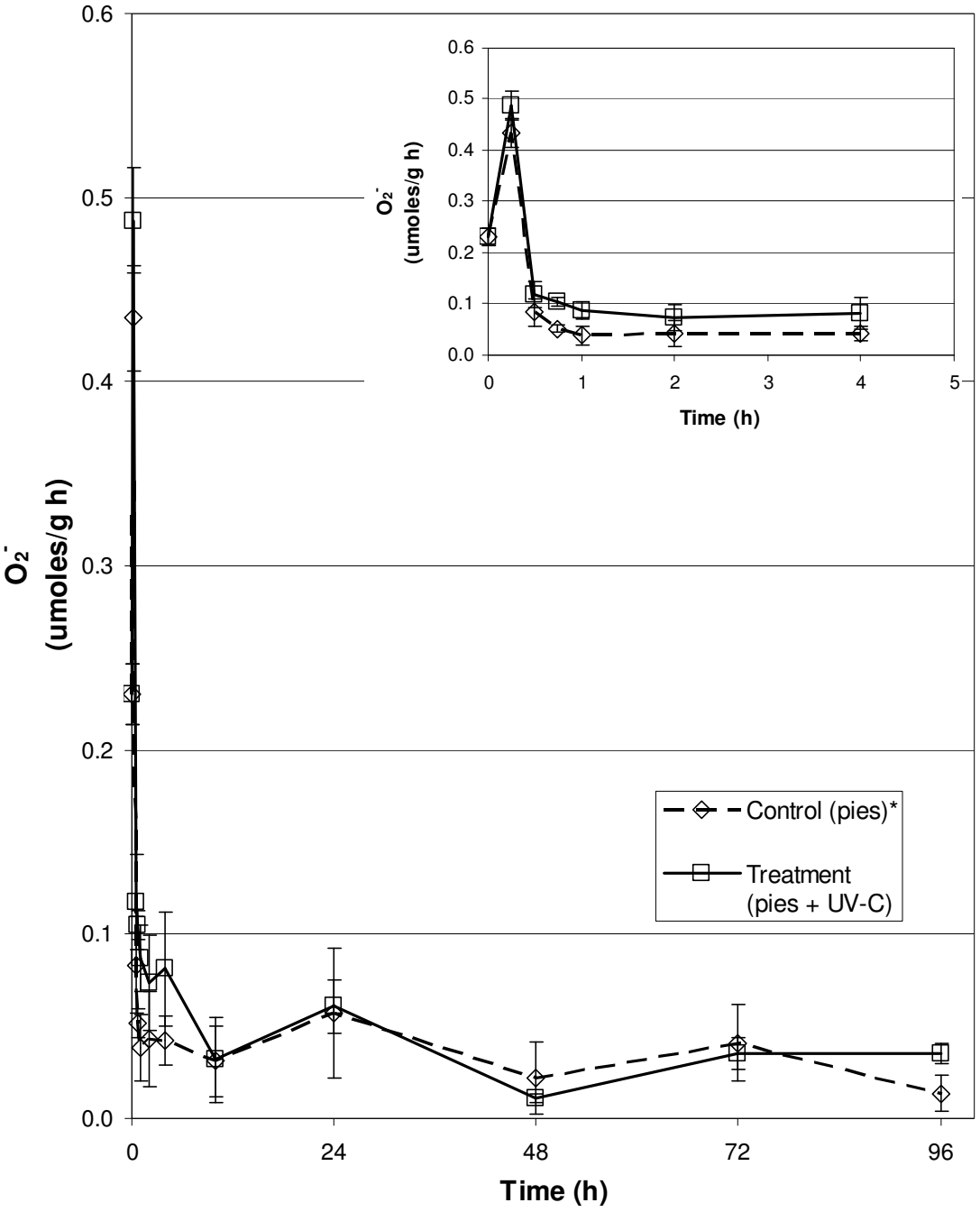

Figure 24 – Superoxide radical production of carrot pies ± UV-C through time. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* Overall means for the control and treatment are significantly different (P<0.05).

Hydrogen peroxide production was monitored through time for both carrot pies and UV-C treated samples (Figure 25). For carrot pies, there was only a slight peak after 45 minutes, then a decreased until reaching steady state. For the UV-C treated samples, the peak value was larger ($P<0.05$), reaching a maximum after 1 h, then decreased slightly for 1 h before a more rapid decline, until reaching steady state after 1 d. Tewari and others (2005) showed a similar trend of higher H_2O_2 production for iron-starved plants compared to the control.

The rapid production and accumulation of ROS causes an 'oxidative burst' which has been demonstrated in wide variety of plant/stress systems (Low and Merida 1996). The functions of this oxidative burst have been proposed to heal damage caused by stress (wounding or pathogen attack), as antimicrobial agent to prevent infections, and to induce expression of defense-related genes (Low and Merida 1996). ROS intermediates, such as O_2^- and H_2O_2 were considered to be toxic by-products, which were disposed of by using antioxidants (Mittler 2002). However, in recent years, ROS has been investigated for its role and participation in stress signaling events. Because of these, plant cells require at least two mechanisms; one that will control low levels of ROS for signaling purposes and the other one that has capability to detoxify excess ROS, especially during stress, so as not to kill the cells (Mittler 2002).

Lipoxygenase activity

Lipoxygenase activity was quantified as an indirect approach for measuring jasmonic acid production caused by wounding and UV-C radiation through time (Figure 26). For both samples, there was a small peak after 10 h reading and then a decrease after 24 hour, but eventually, a gradual increase through time. Barry-Ryan and O'Beirne (1998) showed that lipoxygenase activity in carrot slices increased through time up to 8 d, then the activity remained constant from day 8 to day 10.

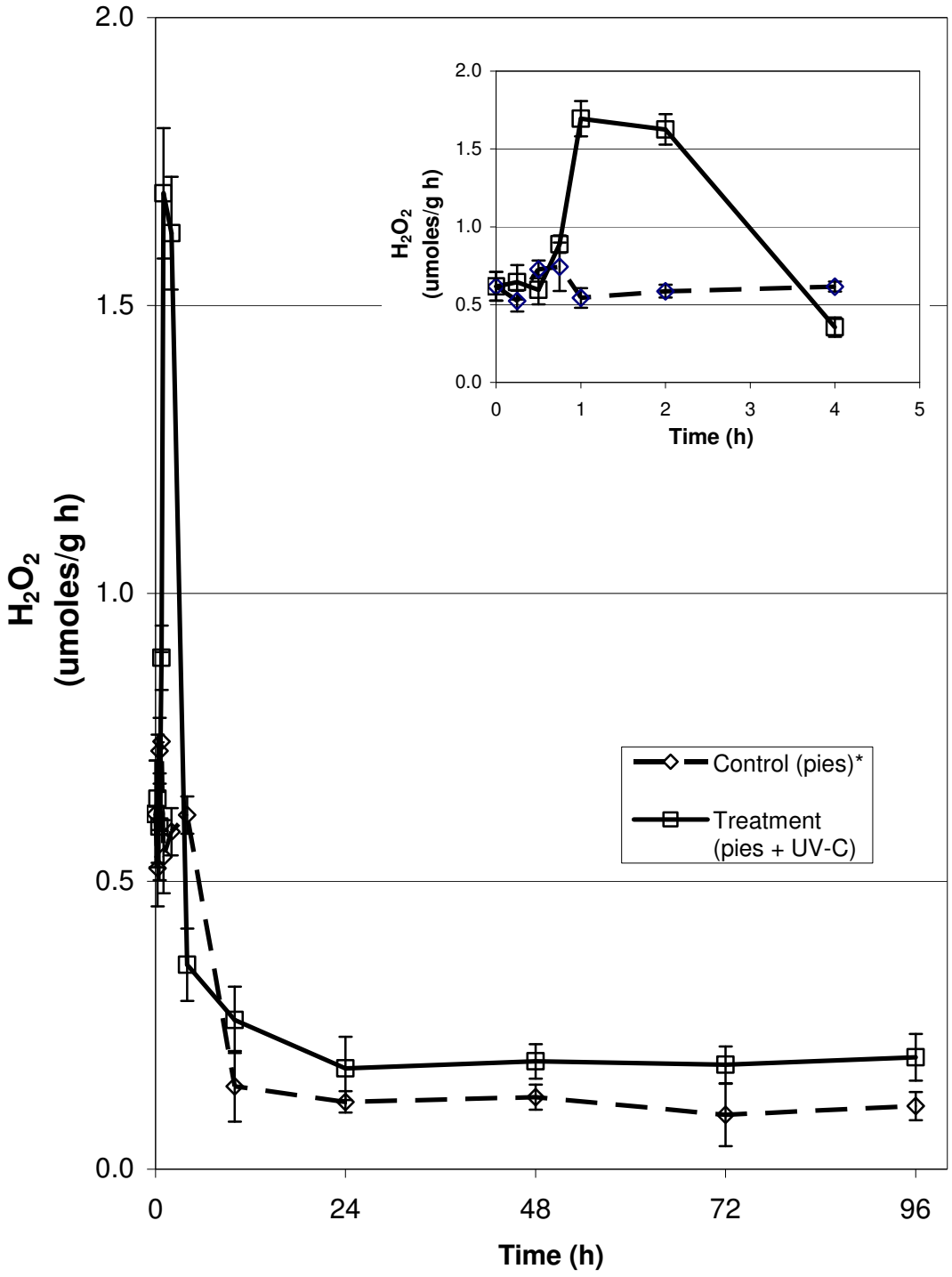

Figure 25 – Hydrogen peroxide production of carrot pies ± UV-C through time. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* Overall means for the control and treatment are significantly different (P<0.05).

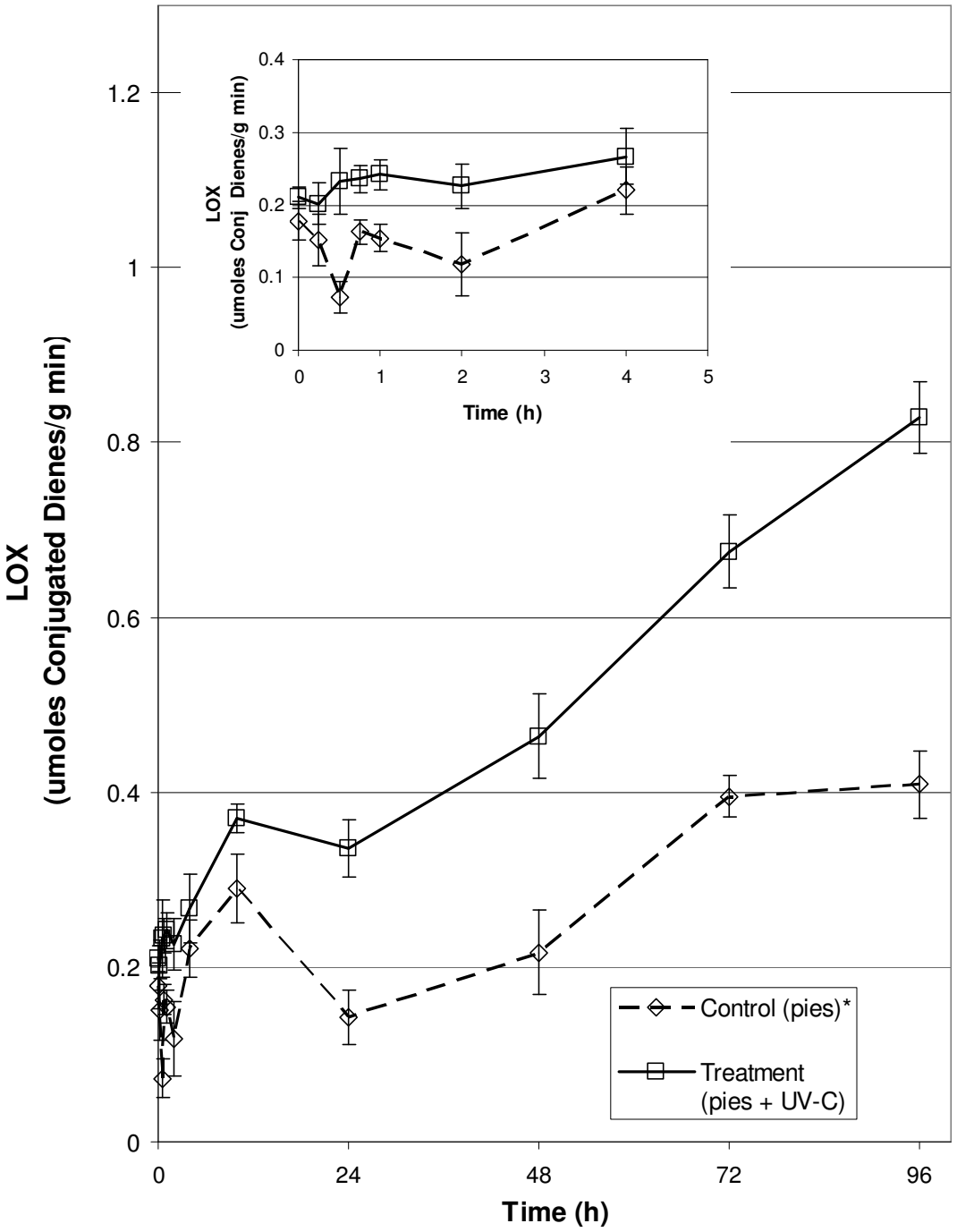

Figure 26 – Lipoxygenase activity of carrot pies ± UV-C through time. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* Overall means for the control and treatment are significantly different (P<0.05).

Karakurt and Huber (2003) reported that LOX activity of fresh-cut papaya fruit stored at 5^{o}C increased gradually, reaching a maximum at day 4. The LOX activity of the fresh-cut papaya fruit samples was also considerably higher compared to the intact fruit.

For all of the readings, the UV-C treated sample was always significantly higher ($P<0.05$) than the control. This is parallel to the result found by An and others (2000) when they compared the LOX activity of spring wheat seedlings. Exposure to UV-B radiation caused LOX activity to have more pronounced increase compared to the controls.

Karakurt and Huber (2003) suggested that wounding caused the release of membrane unsaturated fatty acids that can serve as a substrate for LOX. This process eventually leads to the increase in LOX activity of the wounded samples compared to the controls. Another possible explanation is that wounding and/or UV stresses, send a signal to the cell membrane receptor that induces the *de novo* synthesis of linoleic acid and other unsaturated fatty acids providing more substrate for LOX.

Ethylene production

Ethylene production was measured for both the carrot pies and the UV-C treated samples (Figure 27). There was no significant increase and no difference ($P>0.05$) between the carrot pies and the UV-C treatment within the first hour. After 1 h, the ethylene production of the UV-C treated sample was higher ($P<0.05$) and the controls. Carrot pies reached a maximum ethylene production 4 h after wounding then decreased gradually until reaching a steady state after 24 h. The UV-C treated samples reached a peak after 24 h then decreased gradually until reaching steady state after 72 h storage. An and others (2000) reported that ethylene production in spring wheat leaves was significantly higher in UV-B treated samples compared to a control.

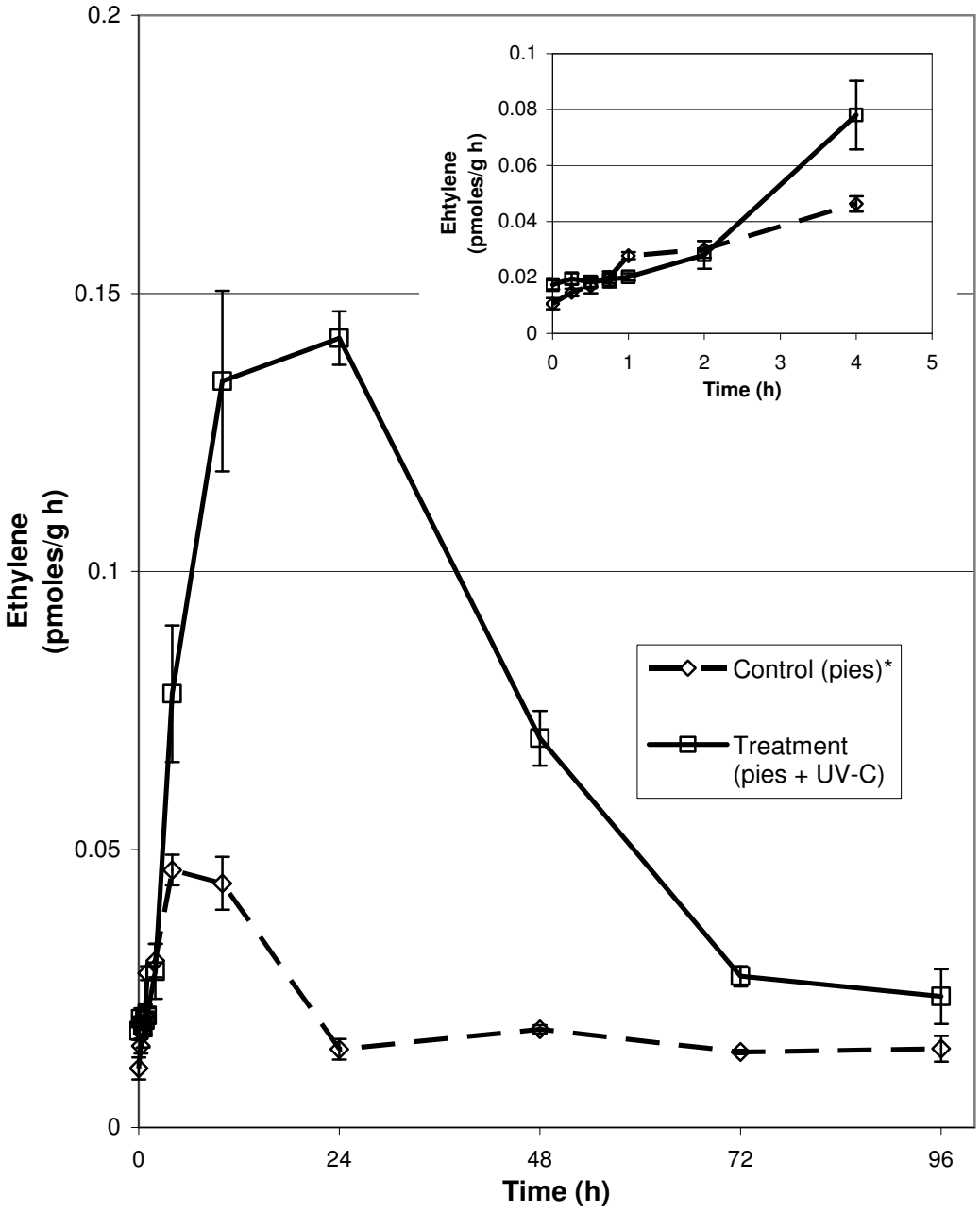

Figure 27 – Ethylene production of carrot pies ± UV-C through time. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* Overall means for the control and treatment are significantly different (P<0.05).

Ethylene production as a result of abiotic stress is one of the most common and marked reaction in plant biology (Morgan and Drew 1997). Abeles (1973) created the term "stress ethylene" to refer to the rapid biosynthesis of the ethylene gas associated with environmental or biological stresses experienced by plants.

ACC (1-aminnoocyclopropane-1-carboxylate) has been identified as the precursor of ethylene and has been suggested that ethylene synthesis rate depends on the change in the activity of ACC synthase enzyme (Morgan and Drew 1997). This enzyme occurs as a multigene family, and each gene is regulated independently, some by stress (Morgan and Drew). Another enzyme in ethylene biosynthesis, ACC oxidase, can also be activated by stress, especially by wounding (Hyodo and others 1993). The use of transcription and translation inhibitors had shown that genes for both ACC synthase and ACC oxidase enzymes are regulated at transcription level (Morgan and Drew 1997). Calcium and protein kinase had been demonstrated to be essential for transduction of stress-ethylene responses (Sessa and others 1996).

Stress-induced signal transduction

The general cellular process and regulation for activating plant secondary metabolite starts with a signal, either extracellular or intracellular, is perceived by a receptor on the surface of the plasma membrane, which then initiates a signal transduction network that leads to activation or *de novo* synthesis of transcription factors to regulate gene expression involved in the plant secondary metabolism (Figure 28) (Low and Merida 1996, Koiwa and others 1997, Morgan and Drew 1997, Taiz and Zeiger 1998, Vranova and others 2002, Zhao and other 2005). This signal transduction is a complex network with multiple sequential reactions to establish an efficient defense mechanism (Zhao and others 2005). It has several components that consist of some parallel or cross-linking signaling pathways that eventually lead to different target responses. An improved knowledge of signal transduction will be beneficial in developing strategies to modify the production of target compounds by either activation or inhibition of certain metabolic pathways (Zhao and others 2005).

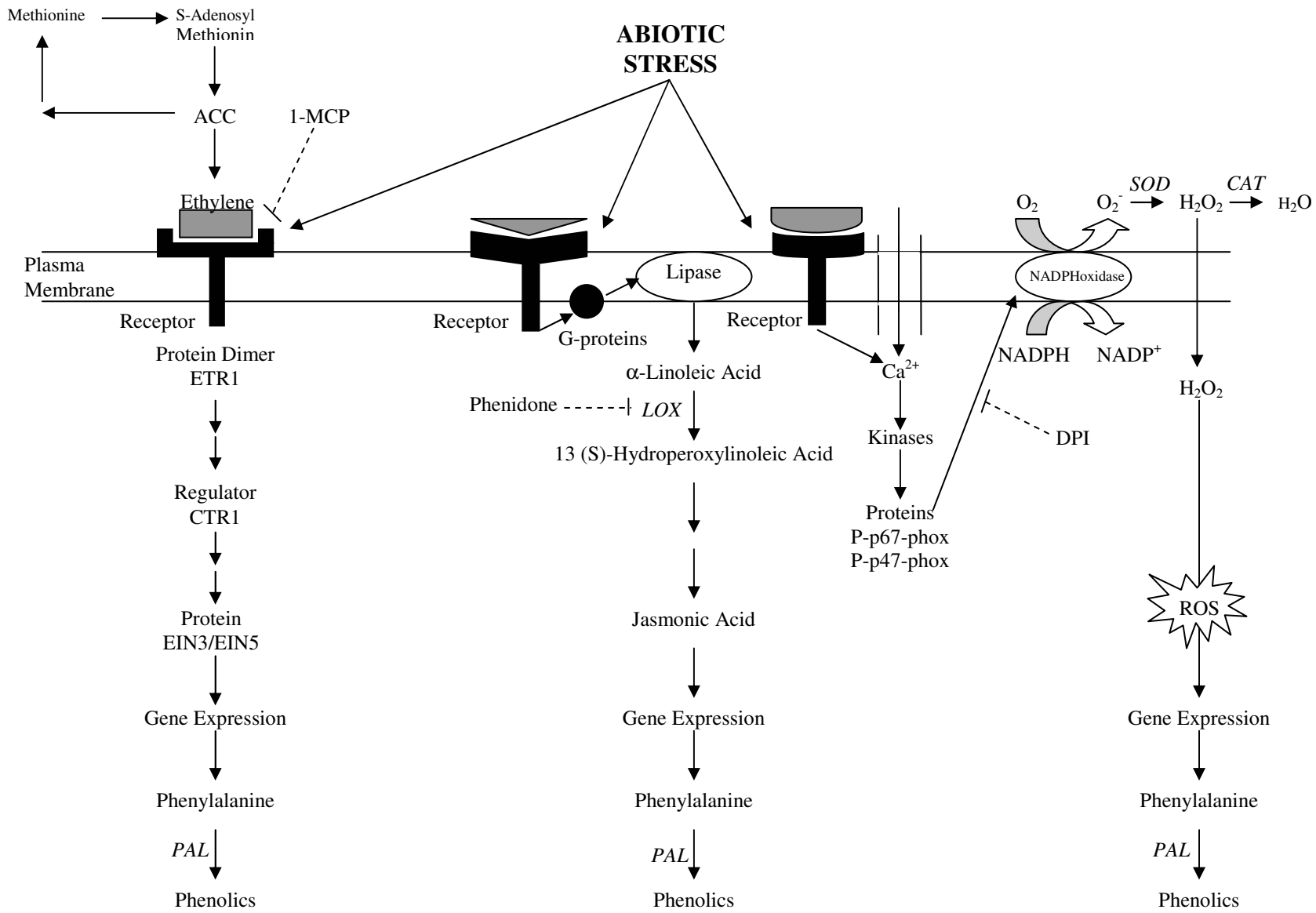

Figure 28 - Stress-induced signal transduction (adapted from Low and Merida 1996, Koiwa and others 1997, Morgan and Drew 1997, Taiz and Zeiger 1998, Vranova

Remarkable progress has been made in understanding how plants perceive and respond to ethylene (Morgan and Drew 1997). A complex network of genes has been identified: ETR1/ETR4 ⟶ CTR 1 ⟶ EIN2 ⟶ EIN3/EIN5 ⟶ responses (Morgan and Drew 1997, Taiz and Zeiger 1998). Some of these genes are negative and some are positive regulators. When a plant experienced a stress, first, the plant must detect and measure the intensity of the stress. After the stress is perceived, a signal is sent to the receptor and transduced to synthesize the production of ethylene, which eventually leads to observable symptoms (Morgan and Drew 1997). However, not much is understood about the mechanisms of stress perception and although interactions with several other plant hormones have been suggested, there is no study where the entire series of events can be traced from stress perception to ethylene response (Morgan and Drew 1997).

Another plant hormone that has been observed to increase as a result of stress is jasmonic acid (Wasternack and Hause 2002). This compound is derived from the octadecanoid pathway and originated from α-linoneic acid. Oxygenation by lipoxygenase (LOX) at carbon atom 13 resulted in (13S)-hydroperoxy linoleic acid, which then converts to cis (+)-12-*oxo*-phytodienooic acid (OPDA) by the action of allene oxide synthase (AOS) and allene oxide cyclase (AOC) (Wasternack and Hause 2002). All of these processes eventually lead to the formation of (+)-7-*iso*-jasmonic acid. There are two parts of stress-induced jasmonic acids formation: (1) early gene activation, mainly local generation of signals in the vascular bundles which lead to both local and systemic responses and (2) late gene activation, preferentially defense related gene expression in the mesophyll cells upon activation in the systemic tissue by a systemic signal (Wasternack and Hause 2002).

As an aerobic organism, plant needs oxygen to produce energy. During the reduction of O_2 to H_2O, ROS such as O_2^-, H_2O_2, and hydroxyl radical can be formed (Vranova and others 2002). When stress occurs, Ca^{2+} influx, alkalinization of the apoplast, protein phosphorylation and translocation of the p-47-phox and p-67-phox subunits lead to the synthesis of NADPH oxidase and triggers the oxidative burst (Low and Merida 1996, Vranova and others 2002). NADPH oxidase is considered to be the main source of the early and sustained accumulation of ROS (Vranova and others 2002). NADPH oxidase is responsible for the reduction of O_2 to O_2^- and with the presence of SOD enzyme, O_2^- is then converted to H_2O_2 (Low and Merida 1996, Zhao and others 2005). Both O_2^- and H_2O_2 have been shown to be involved in plant stress-induced defense responses, either locally or systemically. Expression of genes related to plant defense pathway has been demonstrated as consequences of pathogen attack, chilling injury, wounding, and excess light (Vranova and others 2002).

The results (Figures 21-26) suggested that there is a series of outcomes generated as a result of abiotic stress and the response is more significant when the tissue endures a combination of multiple stresses. Following the general signal transduction process described above, the result of the present study shows that wounding and UV radiation stresses induce several signaling mechanisms. The first event which happened immediately after the stress was production of ROS (O_2^- and hydrogen peroxide productions) because the peaks occurred at 15 min to 1 h. The next event was the ethylene synthesis, which had a peak at 4-10 h, then follows by activation of LOX enzyme activity which is an indication of the increase in the JA production. After a series of cascading reactions induced by the signaling molecules, the cell started to elicit transcription factors to regulate gene expression of PAL enzyme, which eventually synthesized phenolic compounds.

The use of inhibitors in the phenolic synthesis of shredded carrots

Total phenolics for all the treatment are significantly higher ($P<0.05$) compared to the initial day (Figure 29). All inhibitors showed a dose response effect by decreasing

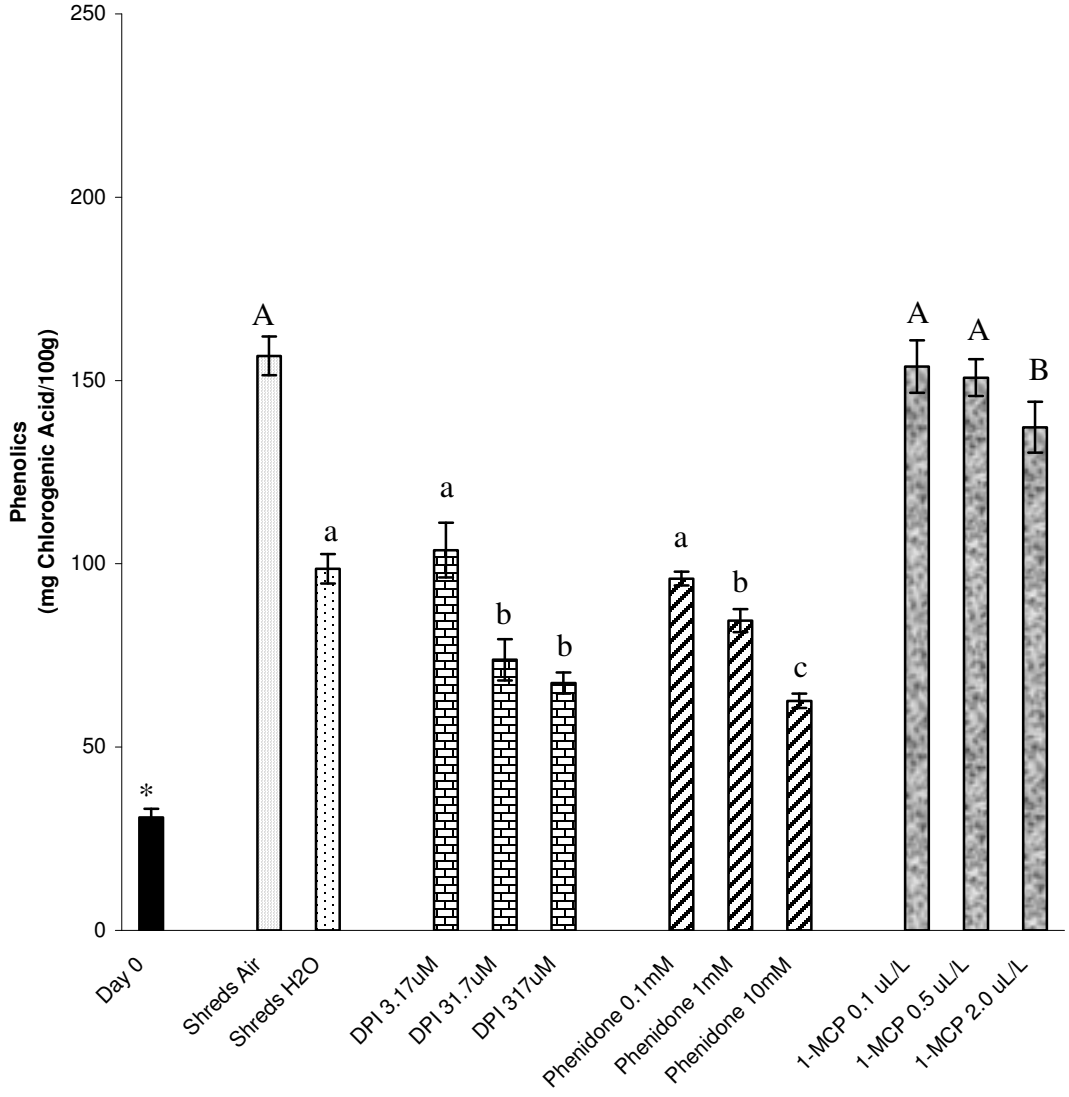

Figure 29 – Total phenolic content of shredded carrots applied with inhibitors of different signaling molecules. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* All treatments are significantly different than day 0 (P<0.05).
abc Treatments with different letters are significantly different than the water control (P<0.05).
ABC Treatments with different letters are significantly different than the air control (P<0.05).

total phenolic synthesis of wounded carrots, indicating that ROS, JA, and ethylene are involved as stress-induced phenolic signaling molecules.

The first two concentrations of 1-methylcyclopropene (1-MCP), 0.1 and 0.5 μL/L, were not significantly different ($P>0.05$) than the shreds air control. Only the samples applied with 2.0 μL/L showed a decrease ($P<0.05$) compared to the air control. This means that ethylene plays a role in the stress-induced phenolic synthesis.

The shredded carrots dipped in diphenyleneiodonium chloride (DPI) or phenidone were compared with control carrot shreds dipped in water as the control. This is done since there is a significant difference ($P<0.05$) between the air and water controls shredded carrots. It seems there is a "wash-off" effect when the carrots were incubated in the inhibitor solutions. The phenolic content of shredded carrot samples dipped in the lowest DPI concentration (3.17 μM) was not significantly different than the water control shreds. When the carrots were dipped in the higher DPI concentrations of (31.7 and 317 μM) there was a significant decrease ($P<0.05$). The results indicated that ROS plays an important role in the production of phenolics induced by wounding. However, inhibiting ROS is not completely suppressing the wound-induced phenolics production, indicating that either there is other signaling molecule that could be involved or the concentration DPI used was not enough to inhibit ROS completely.

When shredded carrots were treated with phenidone, the total phenolics content decreased ($P<0.05$) compared to water control shreds, with the exception of the lowest phenidone concentration. These results indicate that JA also plays an important role in the production of phenolics caused by wounding stress. However, inhibiting JA alone does not inhibit the phenolic production entirely. It is possible that ROS and JA are both equally important and necessary for the wound-induced phenolic metabolism.

The use of inhibitors in the phenolic synthesis of wounded and UV-treated carrot

Figures 30-32 indicate that all treatments had higher phenolic content ($P<0.05$) compared to carrot on the day 0.

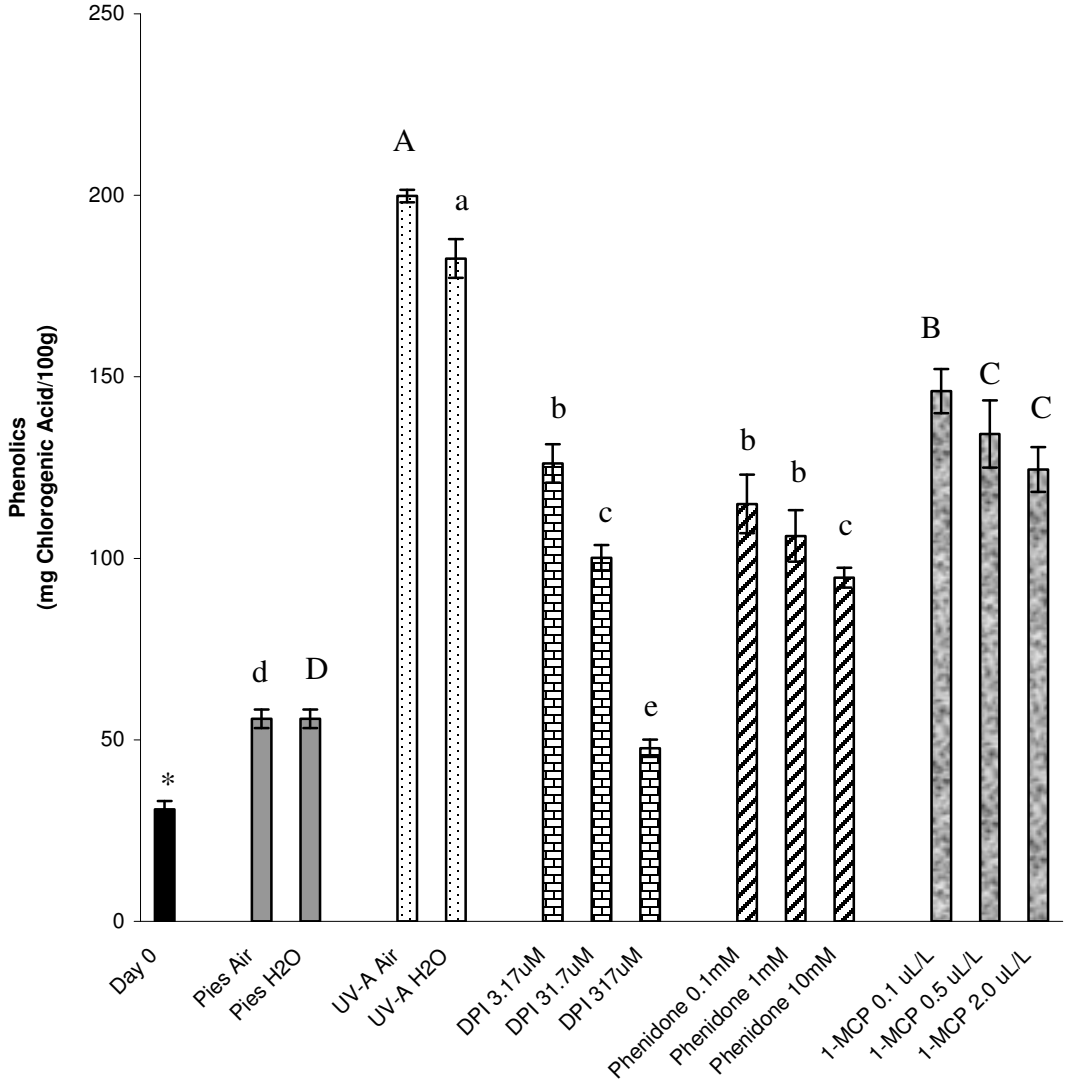

Figure 30 – Total phenolic content of carrot pies radiated with UV-A and applied with inhibitors of different signaling molecules. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* All treatments are significantly different than day 0 (P<0.05).
abcde Treatments with different letters are significantly different than the water control (P<0.05).
ABCD Treatments with different letters are significantly different than the air control (P<0.05).

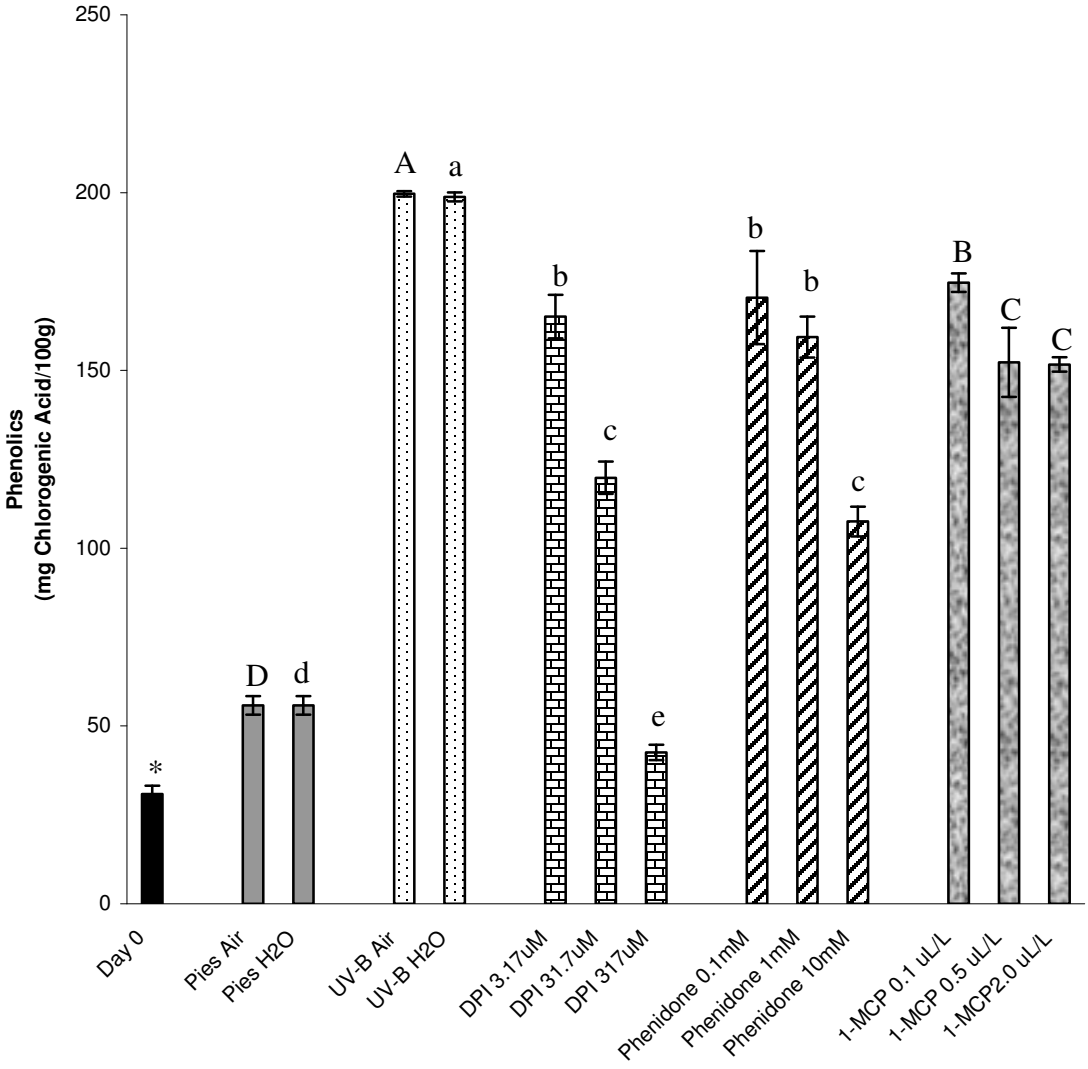

Figure 31 – Total phenolic content of carrot pies radiated with UV-B and applied with inhibitors of different signaling molecules. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* All treatments are significantly different than day 0 (P<0.05).
abcde Treatments with different letters are significantly different than the water control (P<0.05).
ABCD Treatments with different letters are significantly different than the air control (P<0.05).

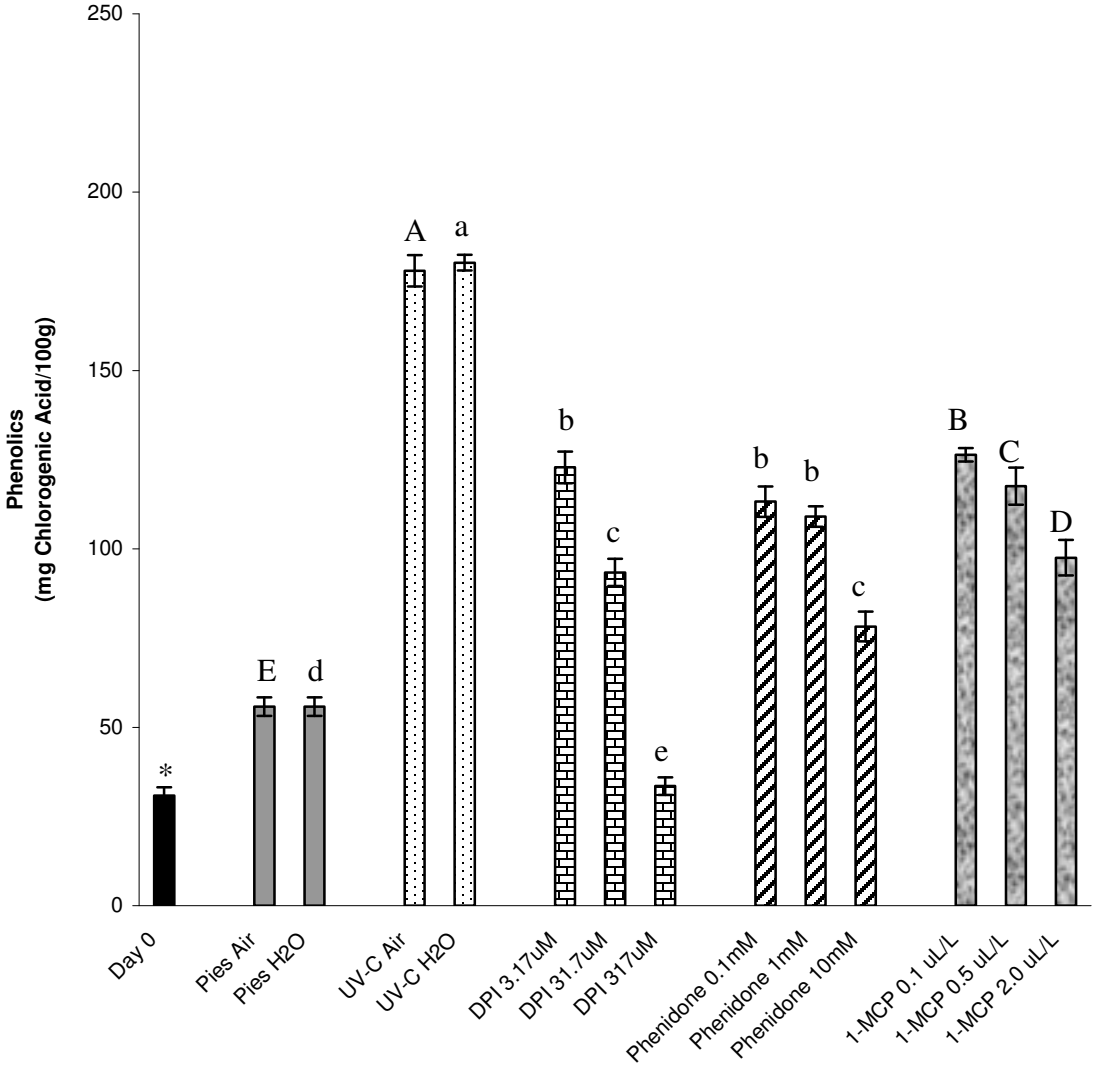

Figure 32 – Total phenolic content of carrot pies radiated with UV-C and applied with inhibitors of different signaling molecules. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

* All treatments are significantly different than day 0 (P<0.05), except for DPI 317 µM.
abcde Treatments with different letters are significantly different than the shreds water control (P<0.05).
ABCDE Treatments with different letters are significantly different than the shreds air control (P<0.05).

These graphs indicate that wounding stress on carrot pies generated an increase in phenolic content and that exposure of carrot pies to UV radiation induced a significant synergistic effect. Unlike the previous result, these data showed that the phenolic contents of the air and water controls carrot pies with and without UV radiation were not significantly different ($P>0.05$) because the wounded surface area is much less in the pies (A/W=6.0 cm^2/g) compared to shreds (A/W=23.5 cm^2/g).

The UV-radiated samples treated with DPI showed a dose response effect by lowering phenolic content ($P<0.05$) compared to the UV water control. The samples treated with the highest DPI concentration shows even lower phenolic content than the water control without UV radiation. This means that ROS is very important in the signal transduction of the phenylpropanoid pathway caused by wounding and UV radiation stresses. The phenolic content of this sample was nearly equal to the initial day value, which indicates that inhibiting ROS will inhibit phenolic production almost completely. It has been shown that hydrogen peroxide is involved as a signal to induce a variety of molecular, biochemical and physiological responses within cells and plants (Neill and others 2002, Vranova and others 2002). According to them, it is likely that H_2O_2 mediates cross-talk between signaling pathways and contributes to cross-tolerance response, which means that exposure of plants to one stress offers protection towards another (Neill and others 2002). Superoxide radical is also an important signaling molecule in plant defensive gene expression of *Arabidopsis* and tomato (Vranova and others 2002).

Figures 30-32 also showed a dose response of phenidone inhibition of the phenolic synthesis induced by wounding and UV radiation stresses. The higher the phenidone concentration, the lower the phenolic contents of the samples.

Comparing to the UV-treated water control carrot pies, the phenolic contents of the phenidone-treated samples were considerably lower ($P<0.05$) but remain significantly higher that the non-UV-radiated water control carrot pies. This indicates that JA elicits UV radiation stress-induced signal transduction network.

Similar trends were also obtained with the ethylene-inhibited samples. The UV-treated carrot pies exposed to 1-MCP generated significantly less ($P<0.05$) amount of phenolics than the UV-treated air control carrot pies. The higher the 1-MCP concentration, the lower the total phenolics. This result indicated that ethylene involved in the signal transduction mechanism of UV stress-induced phenolics. However, blocking the ethylene action did not entirely suppress the phenolic synthesis. One possible reason is that the concentrations used were to low to inhibit the ethylene action, even though the highest 1-MCP concentration applied (2.0 μL/L) to the samples were twice as high as the treatment done in other studies (Fan and Mattheis 2000). According to Blakenship and Dole (2003), the effective concentrations of 1-MCP vary widely with commodity, time, temperature, and method of applications.

Effect of combination of inhibitors on shredded carrots

When we treated the shredded carrots with an individual inhibitor, we observed that by blocking ROS and JA, the total phenolic contents declined significantly ($P<0.05$), about 51-53% less than the stress air control (Figure 33 and Table 10). This data denotes that both ROS and JA are equally important as signaling molecules of wound-induced phenolics. When we blocked ethylene, the total phenolic content was also lower than the control (~ 20%) but not as low as the other two treatments (Table 10).

When carrot shreds were inhibited for ROS and JA (by DPI + phenidone), there was a significant decrease ($P<0.05$) of 46% (Table 10) compared to shreds air control. However, there was no significant difference ($P>0.05$) in total phenolic content compared to the water control shreds (4'), individual DPI and individual phenidone treatments. This could indicate that there is a cross-talk between ROS and JA in the stress-induced phenolic metabolism. Relationship between LOX activity and superoxide radical production in a senescing plant tissue has been discussed by Lynch and Thompson (1984). The treatment with a LOX inhibitor showed a parallel reduction in the enzyme activity and the O_2^- radical formation.

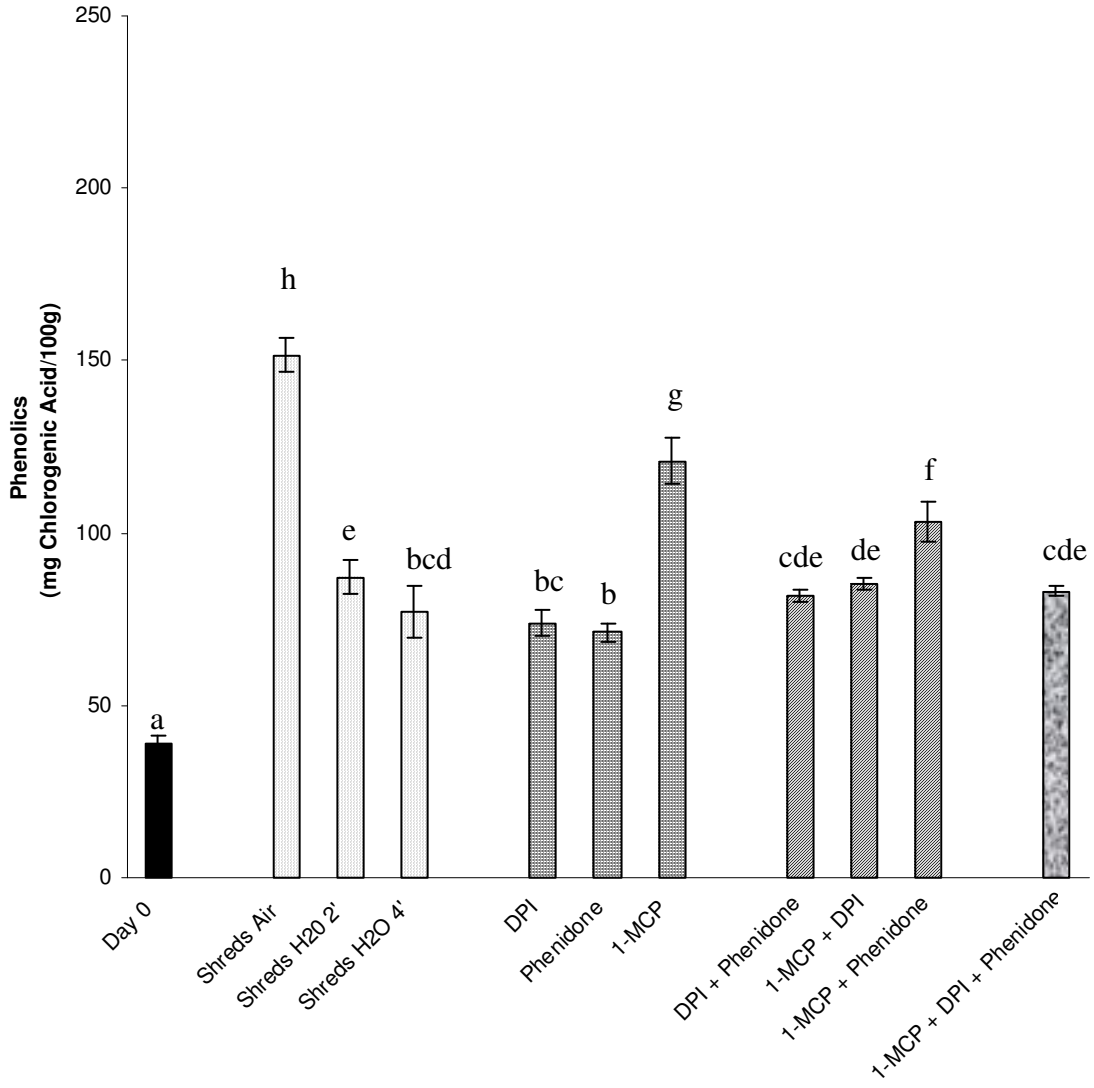

Figure 33 – Total phenolic content of shredded carrots applied with inhibitors of different signaling molecules, either individual or in combinations. The concentrations used were: DPI 317 µM, Phenidone 10mM, and 1-MCP 2.0 µL/L. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

[abcdefgh] Treatments with different letters are significantly different (P<0.05).

Table 10 – Percentage decrease in total phenolics of different treatments with inhibitors compared to the relative stressed air control, based on Figures 33, 35-37.

	DPI	Phenidone	1-MCP	DPI + Phenidone	1-MCP + DPI	1-MCP + Phenidone	1-MCP + DPI + Phenidone
Shreds	51	53	20	46	44	32	45
Pies + UV-A	62	42	19	68	48	34	55
Pies + UV-B	56	26	16	71	42	41	52
Pies + UV-C	64	53	38	71	70	55	60

According to Zhao and others (2005), H_2O_2-mediated lipid peroxidation can initiate the octadecanoid pathway leading to biosynthesis of JA and other oxylipins, which have been reported to induce plant secondary metabolites (Thoma and others 2003). Oxylipins are bioactive compounds which are generated by oxidative catabolism of polyunsaturated fatty acids by coordination action of lipases, LOX, a group of cytochromes P450, and hydroperoxide lyase (Zhao 2005). Most of plant synthesis of oxylipins is caused by injuries and environmental stresses.

The production of this group of compounds can also be triggered by ROS (Zhao and others 2005). Other studies showed that ROS is upstream of JA accumulation and both ROS and JA are key signal components in the stimulation of the secondary metabolite, taxol, produced in *Taxus chinensis* cells (Wu and Ge 2004).

Compared to the air control shreds, total phenolic of the treatment with 1-MCP + DPI decreased by 44% (Table 10).When we inhibited ethylene and jasmonic acid (treatment with 1-MCP + phenidone), the total phenolic content decreased by 34% compared to the shreds air control shreds (Table 10). This indicates that ethylene together with ROS or JA participates in the stress-induced phenolic metabolism.

Interestingly, the result from the treatment of all three inhibitors (1-MCP, DPI and phenidone) shows that total phenolic content is not totally suppressed to the initial day value. Comparing to air control shreds, the total phenolics of this treatment decreased significantly by about 45%, which is very similar to the treatments of DPI + phenidone and 1-MCP + DPI. The interactions among these three inhibitors are not known. Another probable explanation is that there is an unknown factor involved in the stress-induced phenylpropanoid pathway that we did not consider. All of these possibilities need to be investigated in the future.

We propose a diagram that could describe the general wound-induced signal transduction pathway leading to the production of phenolic compounds (Figure 34). A defensive cellular process usually is not regulated by only one signaling pathway, but typically involves one or more pathways that collaborate and regulate the process. Cross-talk among several signaling pathways is an important mechanism in the plant signal transduction network (Zhao and others 2005). The connections between multiple signaling pathways occur at all levels (Scheel and Wasternack 2002), from signal molecules, transcription factors, second messengers, gene expressions to the activation of enzymes to produce different metabolites. It is believed that cross-talk among these pathways enables plants to activate different sets of genes in different situations when the plants are exposed to a stress or a variety of stresses (Zhao and others 2005).

Interaction of JA and ethylene has been shown in many studies. JA signaling can lead to ethylene production in several plants, such as tomato, Arabidopsis, and tobacco (Zhao and others 2005). Our result showed that inhibiting ethylene action with 1-MCP, will inhibit the synthesis of phenolic compounds, which indicates that ethylene involves as one of the signaling molecules.

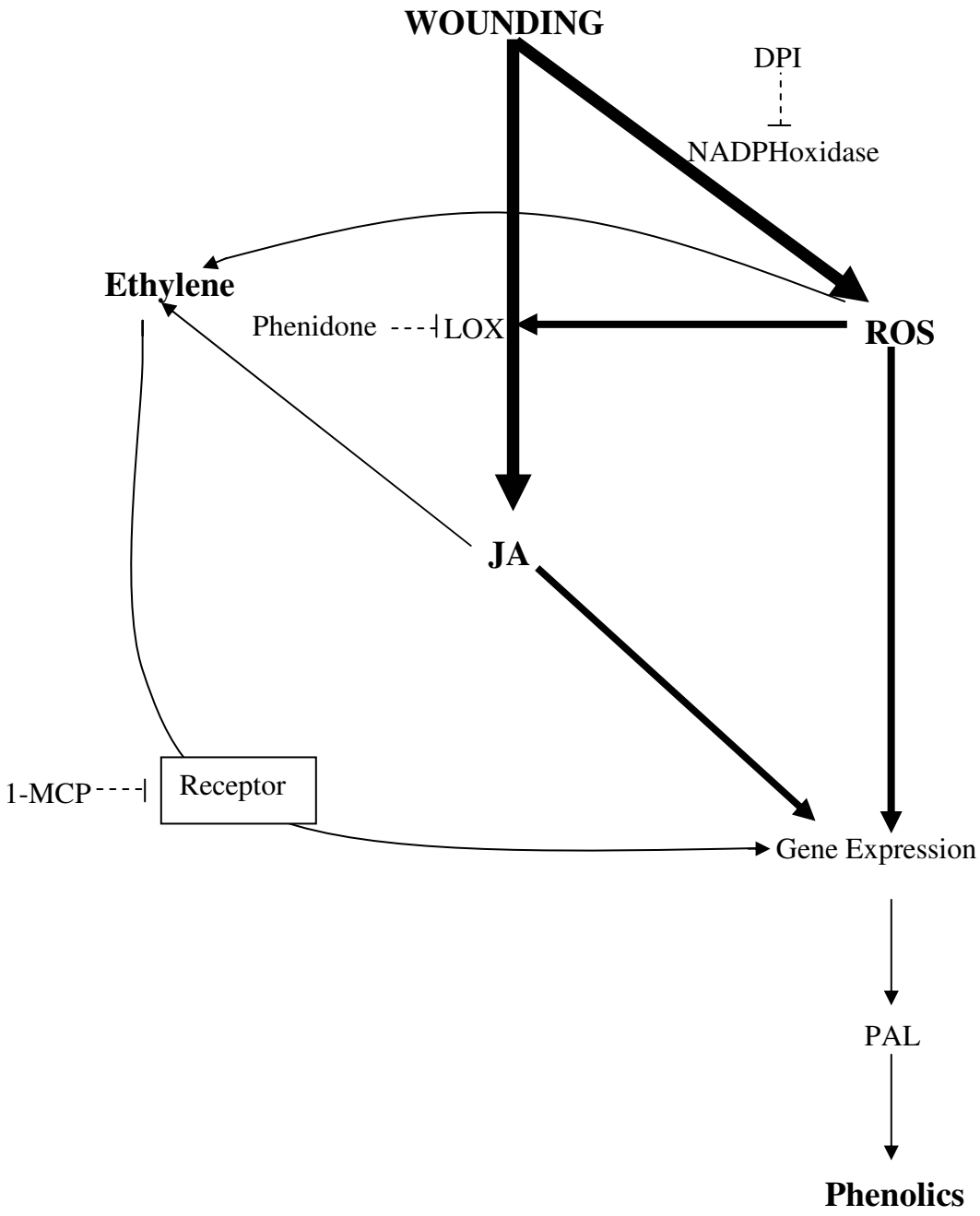

Figure 34 – Proposed signaling network of phenolic synthesis induced by wounding.

However, it is possible that the role of ethylene would only be important when ROS and/or JA are present. Watanabe and others (2001) demonstrated that the expression of wound-induced ethylene gene, *CM-ACS1*, of the fruit of winter squash is induced initially by ROS and then by JA. This means that a response generated by ethylene upon wounding stress is regulated by both ROS and JA. Also, since the total phenolic content decreased equally when we inhibited ROS or JA or combination of both, we proposed that the contribution of ROS and JA as wound-induced signaling molecules is equivalent.

A study on leaves of soybean by Kacperska and Kubacka-Zebalska (1989) suggested that membrane damage promotes ethylene synthesis by accumulation of ACC in the cells as well as activation of lipoxygenase. This enzyme is involved in lipid peroxidation, which is also related to ROS (Lynch and Thompson 1984). Kacperska and Kubacka-Zebalska (1989) also mentioned that lipoxygenase mediates the *in vitro* conversion of ACC synthase to ethylene and also enhances the ethylene production in the microsomal membranes. From our result and information from the literature, we proposed that JA can contribute to the ethylene biosynthesis. This relationship can be regulated by LOX through lipid peroxidation or directly from JA itself.

As mentioned before, cross-talk of multiple signaling pathways is not unusual and is essential for accumulation of plant secondary metabolites and other defense mechanisms, but the mechanism on how the different signaling pathways are integrated into a single cellular process (such as phenolics synthesis) is still not clear. The regulation of cellular processes commonly happens at different levels including transcription, RNA processing and translation, as well as protein phosphorylation (Zhao and others 2005). Even though we identified the signaling components that mediate wound-induced signal transduction to phenolic synthesis, how these events are connected and the mechanisms by which the signals are transduced have yet to be defined.

Effect of combination of inhibitors on carrot pies radiated with UV-A

Figure 35 shows that total phenolic contents of the treatments of individual inhibitor are all significantly lower ($P<0.05$) compared to the UV-A stressed controls. We compared the DPI and phenidone treatments to the UV-A water control carrot pies, while the 1-MCP treatment was compared to the UV-A air control. In this case, we did not do a 4 min dipping time water control since the total phenolic content between the air and water 2 min controls are very similar.

This graph confirmed that all three signaling molecules (ROS, JA, and ethylene) are involved in the stress-induced phenolic synthesis mechanism. However, it seems that ROS is the most important signal molecule, followed by JA, then ethylene. Comparing to the UV-A air control, total phenolics decreased by 62, 42, and 19 %, for treatments inhibited by DPI, phenidone, and 1-MCP, respectively (Table 10).

The combination treatment of DPI + phenidone, resulted a significant decrease in phenolics ($P<0.05$), of about 68% (Table 10) compared to the UV-A control. This value is even lower than the treatments of DPI alone or phenidone alone. Additionally, Figure 35 also shows that by inhibiting ROS and JA, the total phenolics synthesized by UV-A is totally suppressed because the value is not significantly different ($P>0.05$) than the total phenolics of carrot pies non-UV-A treated.

Compared to UV-A stressed control, there is a significant decrease ($P<0.05$), by about 48% (Table 10), in total phenolics when we combined 1-MCP and DPI together to block ethylene and ROS. Moreover, this value is considerably higher ($P<0.05$) than air and water controls carrot pies.

The treatment of 1-MCP + phenidone to inhibit ethylene and JA shows a significant decrease ($P<0.05$) of 34% (Table 10) compared to the UV-A treated controls and was higher ($P<0.05$) compared to the non-UV treated controls carrot pies. The total phenolic value of 1-MCP + phenidone lies in between the total phenolic of the individual treatment (only 1-MCP or only phenidone).

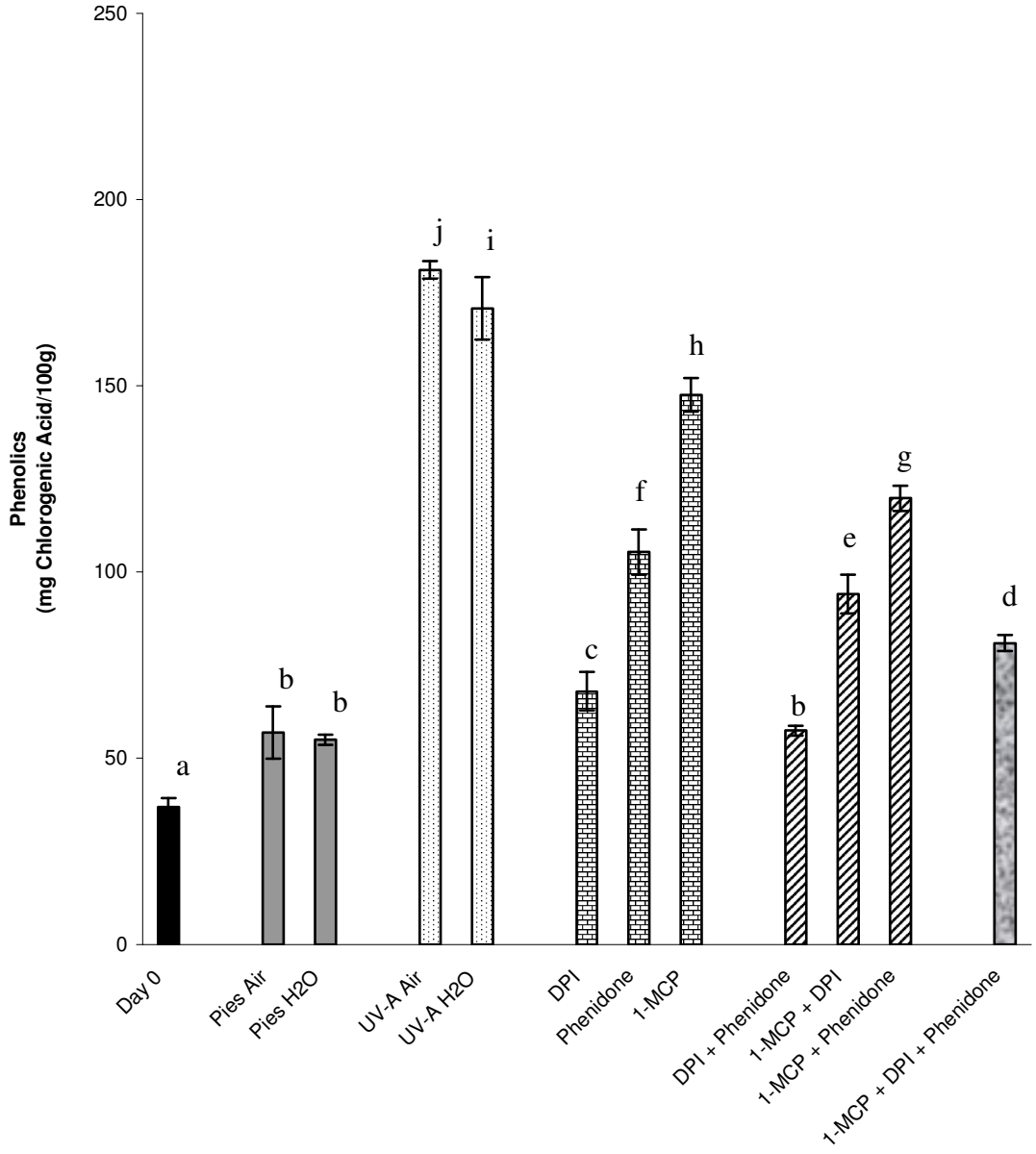

Figure 35 – Total phenolic content of carrot pies radiated with UV-A and applied with inhibitors of different signaling molecules, either individual or in combinations. The concentrations used were: DPI 317 µM, Phenidone 10mM, and 1-MCP 2.0 µL/L. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

[abcdefghij] Treatments with different letters are significantly different (P<0.05).

When we inhibit all three signaling molecules together, the total phenolic content even though significantly lower than the UV-A treated controls (55%; Table 10), it is not as low as the day 0 or even the non-UV-A treated controls carrot pies. This indicates that there could be other signaling molecules induced by the stresses or other possible factors that need to we did not consider. It has been demonstrated that salicylic acid and abscisic acid are involved in activation of stress-induced defensive gene expression in rice (Agrawal and others 2001). Even though these authors did not correlate the hormones directly to the synthesis of phenolics, they mentioned that salicylic acid and abscisic acid have an inverse relationship with jasmonic acid. Therefore, it is possible that when JA was inhibited jasmonic acid, there was an activation of salicylic acid or abscisic acid to produce phenolics. This needs to be investigated and proven in the future.

Effect of combination of inhibitors on carrot pies radiated with UV-B

Radiating the cut carrots with UV-B light synthesized more phenolics (Figure 36). The total phenolics of the carrot pies increased ($P<0.05$) after application of UV-B for 6 h. When stressed carrots was treated with DPI, phenidone or 1-MCP, the total phenolics were all significantly compared to both air and water UV-B controls. Table 10 showed the decrease in total phenolics by 56, 26, and 16% for treatments with DPI, phenidone, and 1-MCP, respectively. This again confirmed that ROS, JA, and ethylene are involved as signaling molecules of stress-induced phenolic metabolism. However, the total phenolics of UV stressed carrot pies treated with inhibitors were still significantly higher compared to control carrot pies. This means that applying only one inhibitor was not enough to completely suppress the stress-induced phenolic production. As mentioned before, it is likely that there is a complex network of multiple stress-induced signaling mechanisms participating.

The treatment of combination DPI + phenidone, showed a phenolic decrease ($P<0.05$), of about 71% (Table 10), compared to the UV-B control. This value was lower than the treatments of DPI or phenidone alone, indicating that there is a synergistic effect of ROS and JA together.

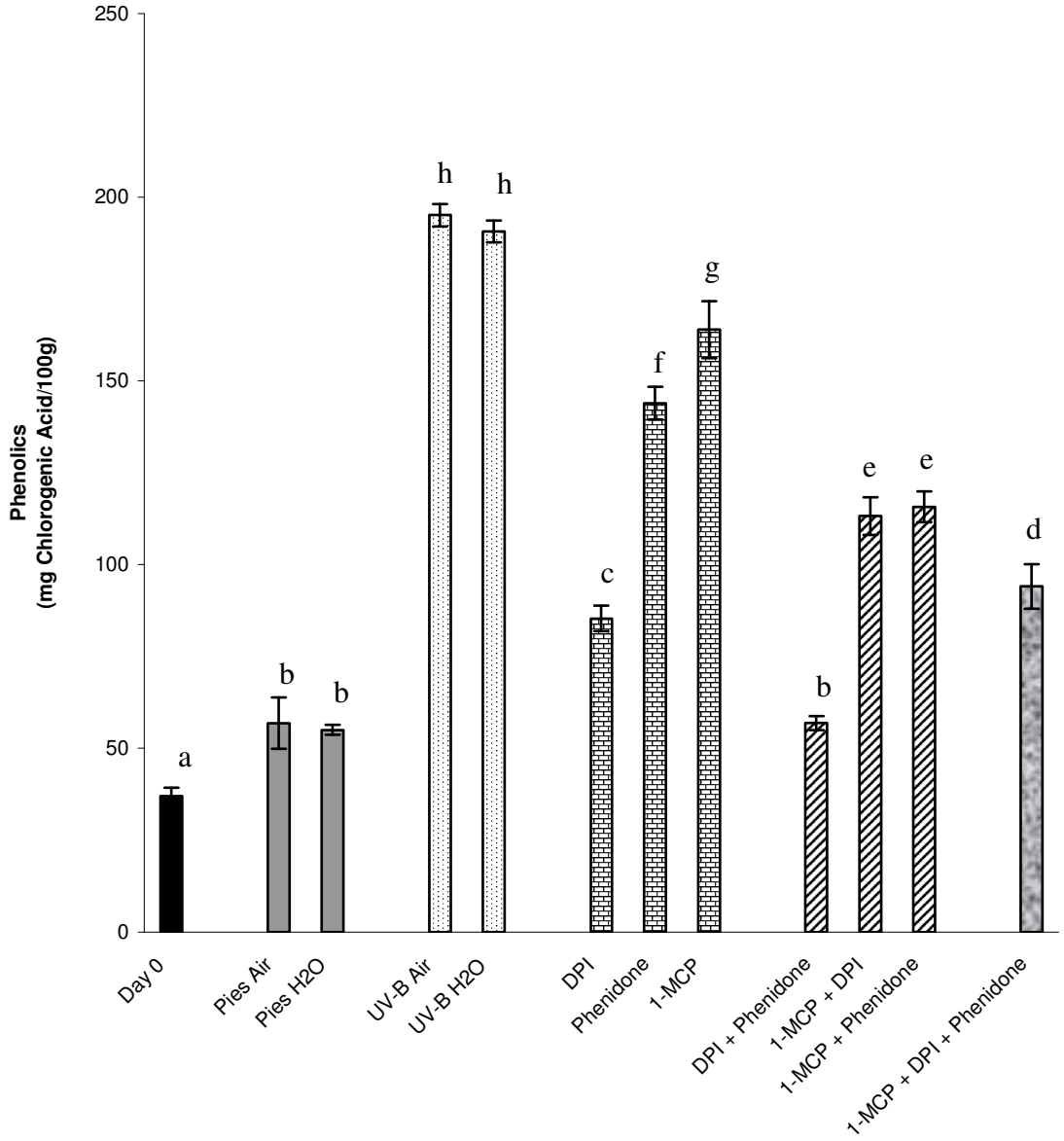

Figure 36 – Total phenolic content of carrot pies radiated with UV-B and applied with inhibitors of different signaling molecules, either individual or in combinations. The concentrations used were: DPI 317 µM, Phenidone 10mM, and 1-MCP 2.0 µL/L. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

[abcdefgh] Treatments with different letters are significantly different (P<0.05).

Furthermore, this showed that by inhibiting ROS and JA, the total phenolics synthesized by UV-B was completely suppressed because the value was not different ($P>0.05$) to the total phenolics of non-UV-B treated carrot pies.

The treatment with 1-MCP + DPI was not different ($P>0.05$) compared to the 1-MCP + phenidone treatment. The decrease in total phenolics compared to the UV-B air control is about 41-42% (Table 10). In the case of 1-MCP + phenidone, the inhibition of the phenolic synthesis was a result of an additive effect.

Inhibiting all three signaling molecules (1-MCP + DPI + phenidone) caused a significant declined in total phenolic content by about 52% (Table 10) compared to the UV-B controls. However, it was still higher ($P<0.05$) compared to the total phenolics of non-UV-B treated controls or the day 0.

Effect of combination of inhibitors on carrot pies radiated with UV-C

Figure 37 shows that treatments of UV-C stressed carrot pies with DPI, phenidone or 1-MCP reduced the total phenolic content ($P<0.05$). Compared to the UV-C air control, the total phenolics decreased by 64, 53, and 38%, for DPI, phenidone and 1-MCP, respectively (Table 10). This means that ROS, ethylene and JA participated in the UV-C stress-induced phenolic metabolism. Similarly to the previous results, ROS is likely to be the primary signaling molecule, follows by JA and ethylene.

The treatment with DPI + phenidone was not different ($P>0.05$) to that of treatment of 1-MCP + DPI. However, the total phenolic content for these two treatments were noticeably lower ($P<0.05$), about 70-71% (Table 10), compared to the UV-C control carrot pies. Additionally, these total phenolic contents were not different ($P>0.05$) compared to the non-UV-C treated control carrot pies. This indicates that combining DPI with phenidone or with 1-MCP is enough to inhibit the phenolic mechanism induced by UV-C radiation. These two treatments also showed lower total phenolic content when compared to treatments that included only DPI, phenidone or 1-MCP.

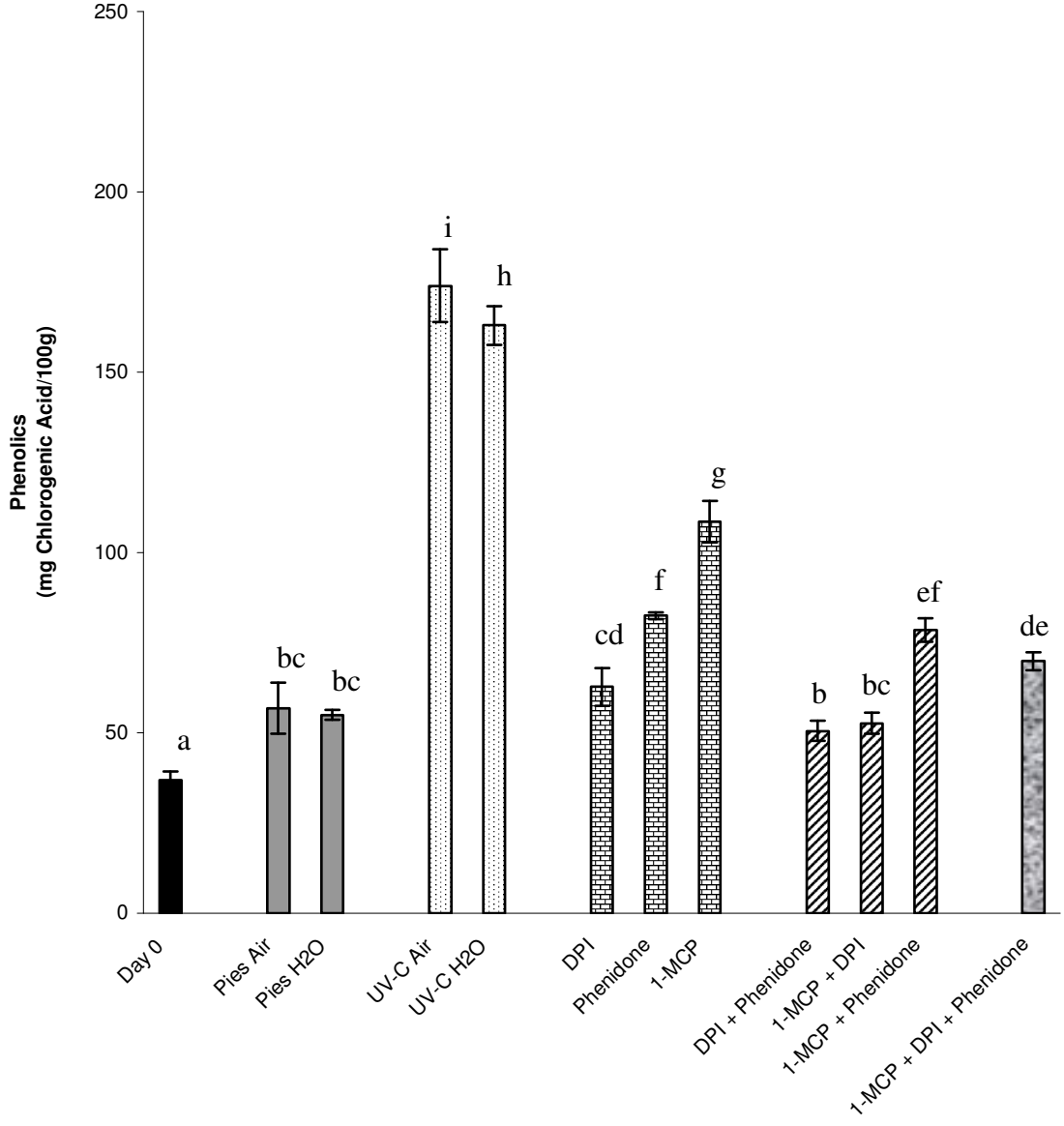

Figure 37 – Total phenolic content of carrot pies radiated with UV-C and applied with inhibitors of different signaling molecules, either individual or in combinations. The concentrations used were: DPI 317 µM, Phenidone 10mM, and 1-MCP 2.0 µL/L. Measurements were taken after 4 d storage at 15°C, except for day 0 reading. All quantifications were on fresh weight basis. Vertical bars represent SD (*n* = 5).

[abcdefghi] Treatments with different letters are significantly different (P<0.05).

The treatment of 1-MCP + phenidone reduced the total phenolic content, by about 55% (Table 10) compared to the UV-C air control carrot pies ($P<0.05$) and was similar to the phenidone alone. Total phenolic content of samples treated with all three inhibitors (1-MCP + DPI + phenidone) decreased ($P<0.05$) by 60% compared to the UV-C treated controls (Table 10), however, this value remained higher ($P<0.05$) compared to day 0 or the non-UV-C treated controls.

We propose a similar diagram to that on Figure 34, which could describe the signaling network of phenolic synthesis when we combine both wounding and UV radiation stresses together (Figure 38). The difference is that with UV radiation, ROS is likely to contribute more than JA or ethylene in the phenolic synthesis.

This diagram agrees with previous reports in the signaling mechanism when a plant is stressed with wounding or UV light (A-H Mackerness and others 1999, A-H Mackerness 2000, Stratman 2003). Both wounding and UV induce the production of ROS, JA, and ethylene, as confirmed in this study.

According to Wang and others (2005), ROS plays essential role in regulating UV-B-induced ethylene in maize seedlings. A proposed signaling mechanism of UV-B by A-H Mackerness (2000) showed that ROS plays a vital role as a second messenger in the induction of pathogen related genes, *PR-1* and *PDF1.2*. The generation of these genes is through JA and ethylene which is promoted from the production of ROS. This founding is similar to the present study, which shows that total phenolic content decreased most significantly when we use DPI to inhibit NADPH oxidase, thus ROS production. Then followed by phenidone, which is LOX (JA) inhibitor and 1-MCP as ethylene action inhibitor. Therefore, on the proposed diagram (Figure 38), it is suggested that ROS is the major signaling molecule elicited by UV radiation and together with JA and ethylene they trigger the expression of phenylpropanoid genes which lead to the activation of PAL enzymes and synthesis of phenolic compounds.

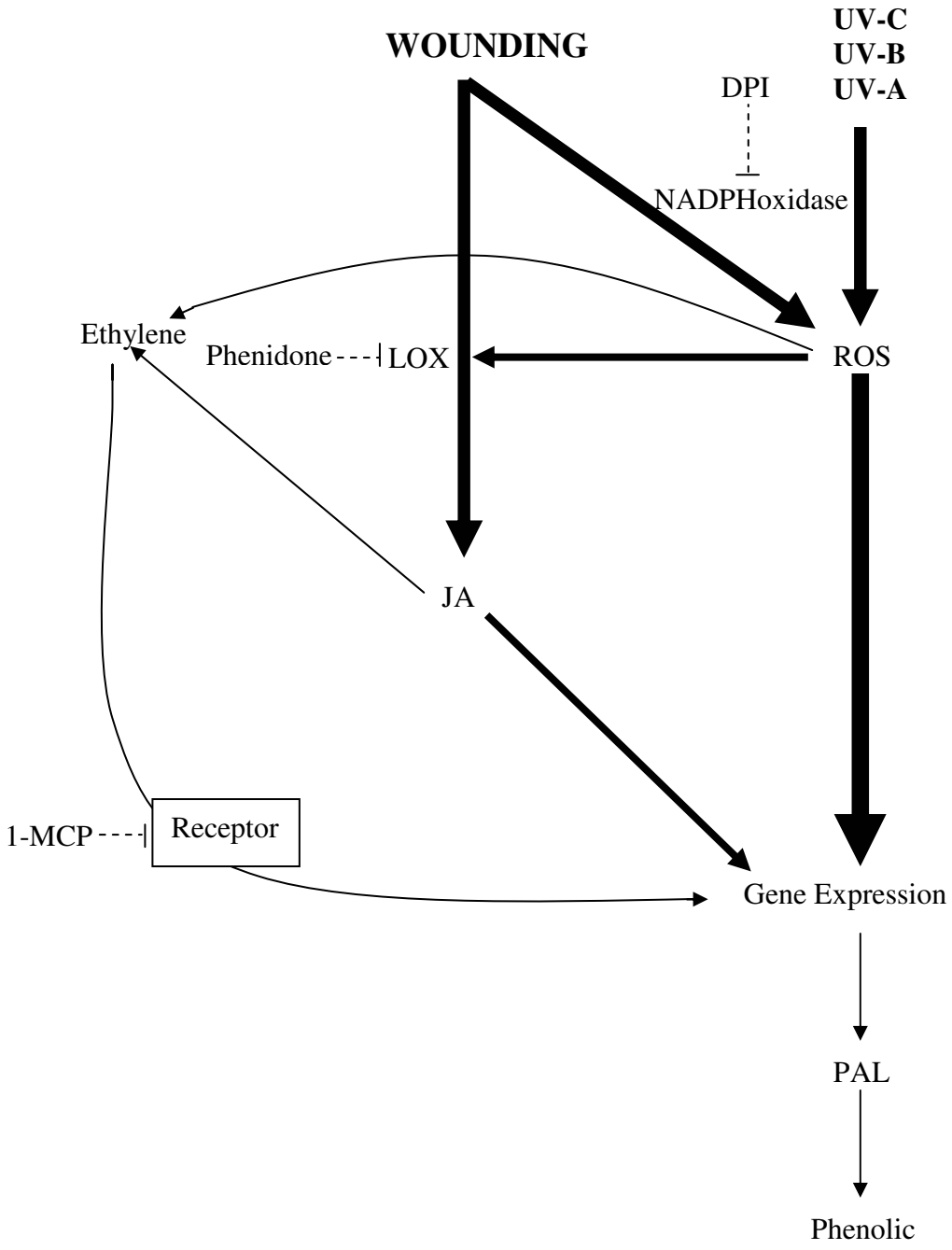

Figure 38 - Proposed signaling network of phenolic synthesis induced by wounding and UV radiation stresses.

Targeting at only one or two signaling pathways, enzymes and genes, or secondary metabolites is not enough to understand the whole pictures of plant secondary metabolism (Zhao and others 2005). It is difficult to obtain a complete view given the complicated metabolic networks, cross-talk of signaling pathways, and various levels of regulatory mechanisms of protein and gene activity (Zhao and others 2005).

Using several inhibitors has helped us identify the related signaling molecules that are involved in the stress-induced phenolic synthesis in carrot tissue. Although this regulation is mainly controlled at enzyme and gene expression levels, a quantitative analysis of total phenolics provides useful information about the interactions and metabolic flow directions. In many pathways with only limited number of genes or enzymes having been characterized, profiling metabolic intermediates is a very direct and important way to understand plant secondary metabolism and regulation (Zhao and others 2005).

Conclusion

Combining wounding and UV-C stress caused synthesis of different compounds in carrot tissue. Starting with the production of ROS (O_2^- and hydrogen peroxide productions), then ethylene synthesis, followed by an increase in lipoxygenase (LOX) enzyme activity. After those events, the cell started to regulate gene expression of PAL enzyme, which in the end proceeded to the production of phenolic compounds.

Using inhibitors for specific pathway showed that JA, ethylene and ROS were all involved in the stress-induced phenolic metabolism. This was demonstrated by a significant decrease in total phenolic contents when phenidone, DPI and 1-MCP were applied. Additionally, different stress induced different signaling molecules and these molecules are likely to be related with each other. For wound-induced stress, the contribution of ROS and JA were equivalent, where as with UV radiation stress, ROS was prone to be the primary signaling molecule, followed by JA, then ethylene.

CHAPTER V

GENERAL CONCLUSIONS AND RECOMMENDATIONS

The application of abiotic stresses such as wounding and UV radiation trigger the synthesis of phenolic compounds in carrot tissue and the duration of storage and wounding intensity will influence the phenolic content, AOX capacity and PAL activity. Results indicated that postharvest abiotic stresses could be beneficial for the food industry to manufacture higher market value products. The fresh-cut produce, food processing, and nutraceutical industries may use these stresses to obtain healthier food with more antioxidant properties. However, these stresses may also cause the tissue to have a shorter shelf-life, thus certain preventive measurement such as lower temperature or appropriate packaging systems will be important to retain the quality for a longer time.

In all of our samples, chlorogenic acid is the main phenolic compound identified. Other phenolics included ferulic acid, *p*-hydroxycinnamic acid and its derivative, chlorogenic acid isomers (3,4- and 3,5-dicaffeoylquinic acids), *p*-coumaric acid derivative, and isocoumarin.

There are several signaling molecules involved in the stress-induced phenolic synthesis. Results show that reactive oxygen species, jasmonic acid, and ethylene are all important signaling molecules. Different stresses seem to undergo different metabolic pathways to induce the phenolics. We proposed different diagrams to illustrate the different pathways involved when applying wounding or a combination of wounding and UV radiation. For wounding stress, the contribution of ROS and JA were equivalent, where as with UV radiation stress, ROS was shown to be the primary signaling molecule, followed by JA, then ethylene.

It will be important to investigate other phenylpropanoid enzymes, such as cinnamic acid 4-hydroxylase (CA4H), 4-coumarate CoA ligase (4CL), chalcone synthase, and other enzymes that are involved in the signal transduction pathway, such

as NADPH oxidase, superoxide dismutase, catalase, and ACC synthase to understand further the stress-induced phenolic signal transduction pathways.

We recommend the study of other factors that could affect the phenolic synthesis, such as temperature, water stress, and other types of light other than UV. Additionally, it will be valuable to include quality parameters measurements, such as color, texture, sugar and vitamins contents, and microbial activity associated with wound-induced and UV-radiated phenolic synthesis. Another interesting study for the future is to determine the effect of controlled atmosphere and modified atmosphere packaging on the stress-induced phenolic compounds and the possibility of using them as tools to increase the shelf-life of the stressed produce.

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VITA

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